Investigation into the aetiology and pathogenesis of inflammatory bowel diseases

Saene, Hendrik Karel Firminus van

H.K.F. VAN SAENE

PATHOGENESIS OF INFLAMMATORY BOWEL DISEASES

lannoo | tielt | bussum

RIJKSUNIVERSTITEIT TE GRONINGEN

INVESTIGATION INTO THE AETIOLOGY AND PATHOGENESIS OF INFLAMMATORY BOWEL DISEASES

PROEFSCHRIFT

ter verkrijging van het doctoraat in de geneeskunde
aan de Rijksuniversiteit te Groningen
op gezag van de Rector Magnificus Dr. L.J. Engels
in het openbaar te verdedigen op woensdag 15 september 1982
des namiddags te 4.00 uur
door

Hendrik Karel Firminus VAN SAENE

geboren te Aalst (België)

PROMOTOR	Prof. Dr. D. van der Waaij
CO-PROMOTORES	Prof. Dr. G.R.A. Vantrappen
	Prof. Dr. W.C. Veeger
REFERENT	Drs. W. Jansen

STELLINGEN

1

The colonization resistance of the digestive tract remains unimpaired in patients with inflammatory bowel disease.

This thesis.

2

The gut associated lymphoid tissue is less active in coating endogenous *Escherichia coli* strains with secretory immunoglobulin A in patients with inflammatory bowel disease.

This thesis.

3

Patients with inflammatory bowel disease are immunologically more responsive to endogenous *Escherichia coli* strains compared with controls.

This thesis.

4

Gastric acid was found not to be a constant barrier to the entrance of organisms into the intestinal tract.

G. Dack, (1934)
J.Inf.Dis.,54:204-220.

5

Anesthesia is known to alter intestinal motility and this factor may affect the microflora.

S. Gorbach, (1967)
Gastroenterology,53:856-867.

6

Sensitivity of bone marrow stem cells for cytostatics increases with endotoxemia.

A. Eaves, (1972)
Ser.Haemat.,5:64-72.

7

The good outcome of an acyclovir therapy for cytomegalovirus infections probably runs parallel with the intensity of concomitant herpes simplex virus infections.

M. Ashraf, (1982)
Lancet,i:173-174.

8

Graft-versus-host disease in allogeneic bone marrow transplantation can be mitigated by injecting bone marrow cells together with cells from Peyer's plaques.

D. Perey, (1972)
Lab.Invest.,27:427-433.

9

Heart transplantation is no longer a clinical experiment but justified patient care.

N. Shumway, (1979)
Br. Heart J.,42:703-708.

10

Eczema is a problem that will only be solved when the word eczema will be expunged from the dermatological lexicon.

A. Ackerman, (1982)
Arch.Dermatol.Res.,272:407-420.

11

De hoge frequentie van infekties door multiresistente stammen in een intensive care unit is in de regel het resultaat van een falend 'klassiek' bacteriologisch beleid.

12

Langdurige beademing als indicatie voor tracheostomie is obsoleet.

13

De bacterie *Pasteurella pestis* heeft met de pandemie van 1348 de aanzet gegeven tot de grootste maatschappelijke revolutie uit de Europese geschiedenis.

14

Het is merkwaardig dat schedels, wervelstormen, verwekkers van epidemieën en practisch alle aërobe staafvormige bacteriën vrouwelijk benoemd worden, hoewel de typisch vrouwelijke eigenschappen ontbreken.

15

Net als in de Islam is in de medische wereld tolerantie voor kritiek door 'ongelovigen' een toevallig wonder.

16

Le déboulonnage de l'amour et de l'érotisme est une entreprise qui peut avoir plus de conséquences qu'on ne l'imagine. Aussitôt qu'on touche à un mythe, tous les mythes sont en danger.

B. Bardot, (1959)
in: *Les écrits de Simone de Beauvoir.*
Gallimard, Paris.

17

Een medisch microbioloog die zich gedraagt als een kamergeleerde hoort niet thuis op een ziekenhuisterrein.

18

Schouderklopjes vormen het pijnlijkste honorarium.

19

All bacteria are equal but some bacteria are more equal than others.

G. Bowel, (1982)
Bacterial Farm
CR Press, Groningen.

Stellingen behorende bij het proefschrift
'Investigation into the aetiology
and pathogenesis of inflammatory bowel diseases'

H.K.F. van Saene,
Groningen, 15 september 1982.

This Ph.D. thesis is based on seven papers.

1. Oropharyngeal flora in inflammatory bowel disease patients.
van Saene, H.K.F., Jansen, W., Veeger, W.C., van der Waaij, D.
J.Med.Microbiol., 1982; in press.

2. Intestinal flora in inflammatory bowel disease patients.
van Saene, H.K.F., Jansen, W., Veeger, W.C., Welling, G.W., van der Waaij, D.
J.Med.Microbiol., 1982; in press.

3. Colonization resistance in inflammatory bowel disease patients.
van Saene, H.K.F., Jansen, W., Veeger, W.C., van der Waaij, D.
Eur.J.Clin.Microbiol., 1982; in press.

4. A novel technique for detecting IgA coated potentially pathogenic microorganisms in the human intestine.
van Saene, H.K.F., van der Waaij, D.
J.Immunol.Methods, 1979; 30:87-96.

5. *In vivo* binding of secretory immunoglobulin A to normal gastrointestinal flora.
van Saene, H.K.F., van der Waaij, D.
Gut, 1982; in press.

6. Evidence for dysfunction of gut associated lymphoid tissue in inflammatory bowel disease patients.
van Saene, H.K.F., Jansen, W., Fidler, V., Veeger, W.C., van der Waaij, D.
Submitted.

7. Prevalence of endogenous *Enterobacteriaceae* spp and of circulating antibodies to endogenous *Enterobacteriaceae* spp in inflammatory bowel disease patients.
van Saene, H.K.F., Jansen, W., Fidler, V., Veeger, W.C., van der Waaij, D.
Submitted.

INVESTIGATION
INTO
THE AETIOLOGY AND PATHOGENESIS
OF
INFLAMMATORY BOWEL DISEASES

Lannoo
Tielt - Bussum
1982

INVESTIGATION INTO THE AETIOLOGY AND PATHOGENESIS OF INFLAMMATORY BOWEL DISEASES

H.K.F. van Saene
Ph. D. Thesis
University of Groningen
(The Netherlands)
1982

A BACTERIOLOGICAL AND IMMUNOLOGICAL INVESTIGATION INTO THE AETIOLOGY AND PATHOGENESIS OF INFLAMMATORY BOWEL DISEASES

Role of local and systemic immunity in translocation of enterobacterial antigens

CONTENTS

CHAPTER ONE

INTRODUCTION

,,intestinal bacteria are not only the major source of infections but they could also be involved in auto-immune disorders''

van der Waaij, D.

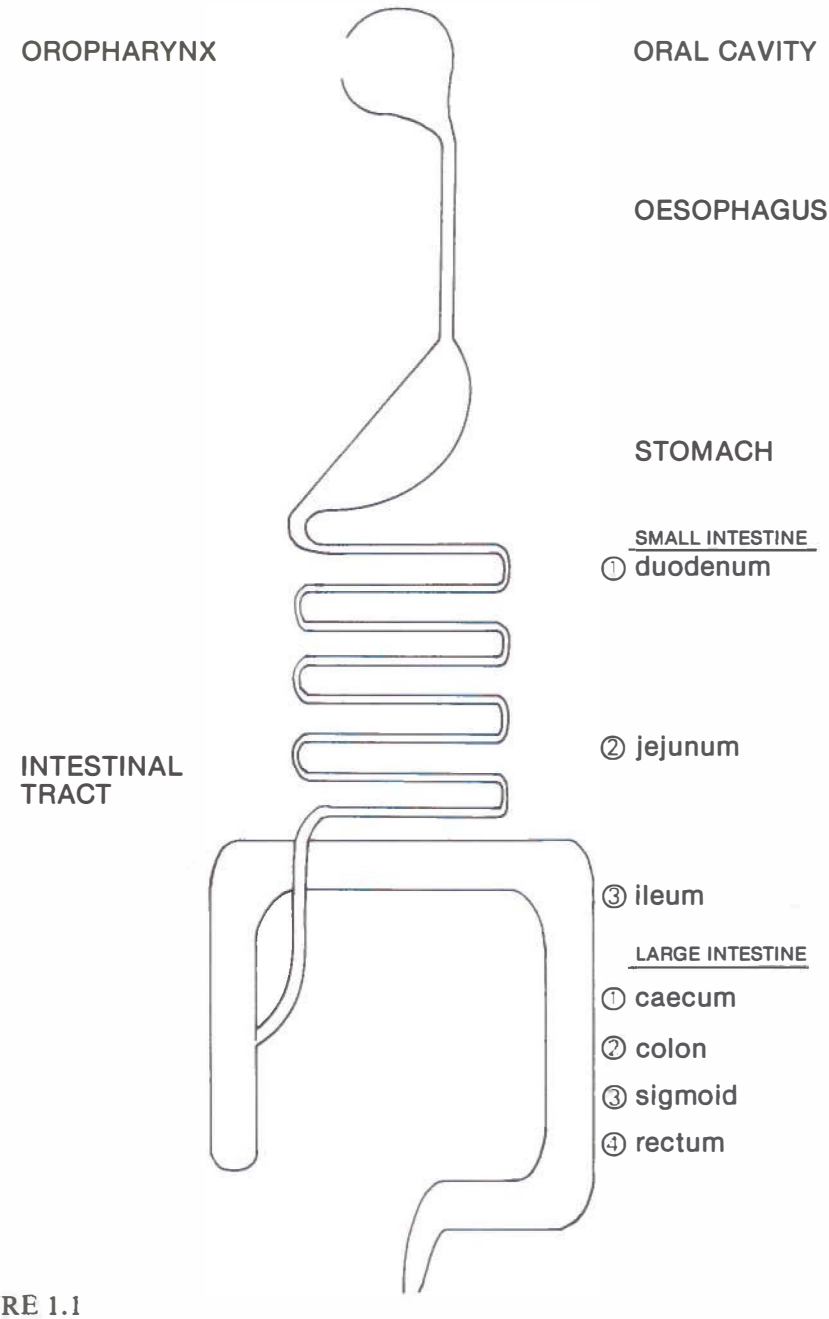


FIGURE 1.1
Crohn's disease is a disease affecting the whole of the digestive tract including mouth and anus. Mostly the affected area are small and large intestine. In contrast, ulcerative colitis is a disease involving the rectum and extending for a variable length of the colon to affect the whole colon.

The diseases collectively known as inflammatory bowel diseases (IBDs) are ulcerative colitis (UC) and Crohn's disease (CD). Both are chronic inflammatory disorders of the digestive tract characterized by a tendency to remission and relapse. They occur in either sex and any age, with peak incidences in the 20- to 40-year age group. In Europe, their incidence amounts to 6 to 9 cases per 100,000 population per annum. Their aetiopathogenesis is poorly understood, certain distressing local and systemic complications are common to the two diseases, and their morbidity and mortality have a severe socioeconomic impact.

Clinically, the IBDs are marked by symptoms as abdominal pain, diarrhoea, fever and weight loss. Besides *intestinal* disorders, both diseases have in common *extra-intestinal* manifestations like arthritis, erythema nodosum, pyoderma gangrenosum, clubbing of fingers, ankylosing spondylitis, iritis, conjunctivitis etc. *Anatomically,* ulcerative colitis is essentially a disease which begins in the rectum; it spreads from the rectum in continuity to involve part or the whole of the large intestine. In contrast, Crohn's disease affects small and large intestine, either singly or in combination. Recently, Crohn's disease is recognized as a disease affecting the whole of the digestive tract, including mouth and anus (Figure 1.1). *Histologically,* the lesion of

Crohn's disease is a transmural one which is predominantly a *submucosal* inflammation. The hallmark of CD is the occurrence of epitheloid granulomata, but these (aspecific) markers are only present in about 60% of patients. Crohn's disease typically affects discontinuous segments of intestine with apparently normal tissue between each 'skip' lesion. Histological abnormalities in apparently normal

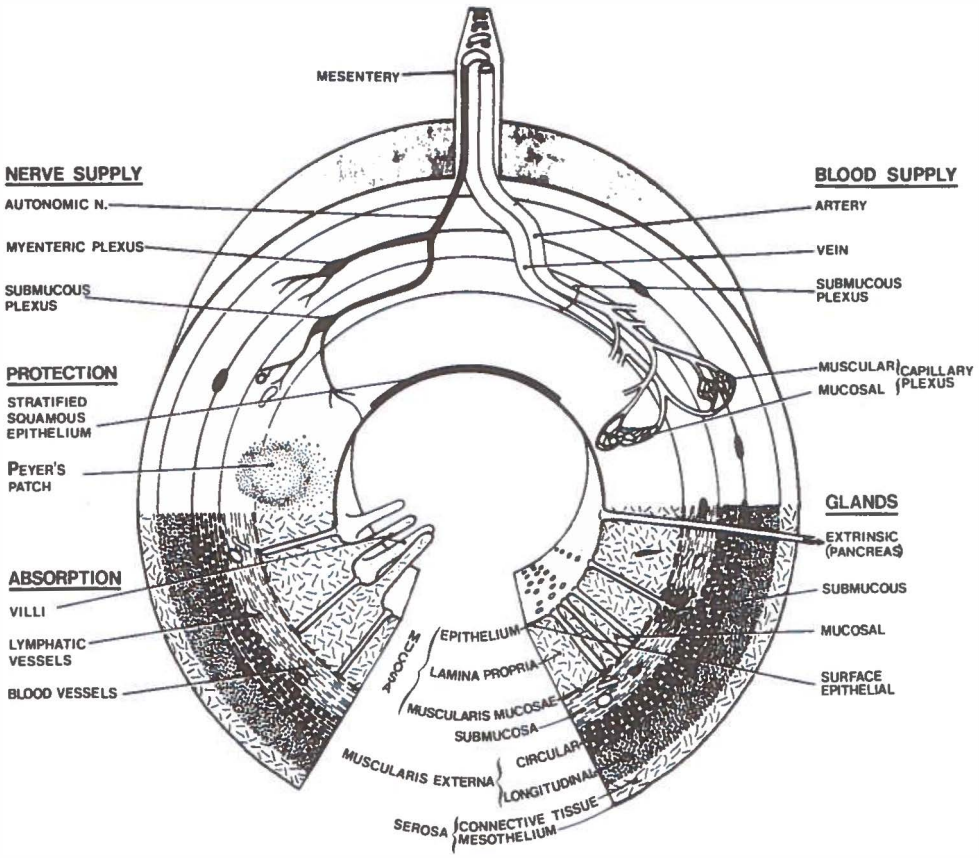


FIGURE 1.2
Cross-section of digestive tract sharing the layers of the wall of the tract. The lesion of Crohn's disease is a transmural one which is predominantly a *submucosal* inflammation (epitheloid granuloma), Ulcerative colitis is primarily a *mucosal* lesion (ulcer). (With permission of Blackwell, Oxford).

unaffected intestine have been reported. Therefore, it may be that the whole of the digestive tract is abnormal and that local factors (such as antigen concentration) determine the site of the overt disease. Unlike Crohn's disease, ulcerative colitis is primarily a *mucosal* lesion (ulcer) which involves the rectum and extends for a variable length of the colon to affect the whole colon in some patients. Crypts abcesses are a typical feature but not a necessary finding (Figure 1.2). Involvement of the terminal ileum can occur in total colitis in continuity with the disease in the colon.

There is now strong view that ulcerative colitis and Crohn's disease may represent the ends of a spectrum with a varying tissue reactivity to a common aetiological agent. This view is supported by the occurrence of typical Crohn's disease and ulcerative colitis in close relatives; the difficulty in distinguishing between the two disorders, both clinically and pathologically, when the colon is affected, and the similar age, race, sex and geographical distribution of the diseases. They have also in common extraintestinal manifestations such as arthritis, iritis and skin lesions. However, there are certain striking differences, notably the frequent involvement of the small intestine and occasionally the upper digestive tract in Crohn's disease, the continuity of mucosal involvement in ulcerative colitis, maximal in the rectum, compared with the characteristic skip lesions in Crohn's disease and the rarity with which classic histological features of ulcerative colitis and Crohn's disease occur in the same individual[1].

This study concerns some facets of the pathogenesis of inflammatory bowel diseases. The investigation focuses on the digestive tract flora and on the role of the local and systemic immunity in translocation of enterobacterial antigens. A series of inflammatory bowel disease patients is compared with a group of healthy individuals. A bacteriological recording of the digestive tract flora and an immunological inventory of both local and systemic immunity (during relapse and remission) are made, in order to examine whether translocation of enterobacterial antigens may be of importance in the pathogenesis of inflammatory bowel diseases. Before formulating the

working hypothesis (Chapter two) we describe in this first chapter the normal (physiological) processes concerning:

(i). bacterial flora of the digestive tract together with an account of control mechanisms (colonization resistance)

(ii). local immunity of the digestive tube controlling entry and invasion of bacterial substances (preventing a systemic immune response).

(iii). systemic immunity (regulation of immune response).

(I)
COLONIZATION RESISTANCE OF THE DIGESTIVE TRACT

Bacterial flora

By far most numerous bacteria colonizing the digestive tract (DT) are the anaerobes. The aerobes to which belongs the family of the *Enterobacteriaceae* only accounts for $<1\%$ of all potentially pathogenic microorganisms (PPMs) inhabiting the digestive tract. The two sites of the digestive tract most easy to sample for culturing are the oropharyngeal cavity (swab or washing) and the intestinal tract (faecal specimen).

1. Oropharyngeal cavity

Besides an enormous quantity and number of anaerobic bacteria, the family of aerobic viridans streptococci colonizes the oropharynx in high growth densities. Healthy adults are in different percentages carriers of *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Haemophilus influenzae* and *Candida albicans. Enterobacteriaceae (Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp etc.) can be present in the oral cavity in a small percentage. The oropharynx is only a transient or temporary residence for *Enterobacteriaceae* spp.

2. Intestinal tract

In the faeces more than 99 % of the bacterial population consist of anaerobes. The aerobic part includes *Enterobacteriaceae,* faecal streptococci and in cases of carriership *Staphylococcus aureus* and *Candida albicans. Streptococcus pyogenes* and *Streptococcus*

pneumoniae as well as *Haemophilus influenzae* colonizing the mouth are — after swallowing — not proof against bile and cannot be cultured from faeces. In contrast to the oropharynx, the intestines normally harbour *Escherichia coli* or other members of the *Enterobacteriaceae* family (Figure 1.3).

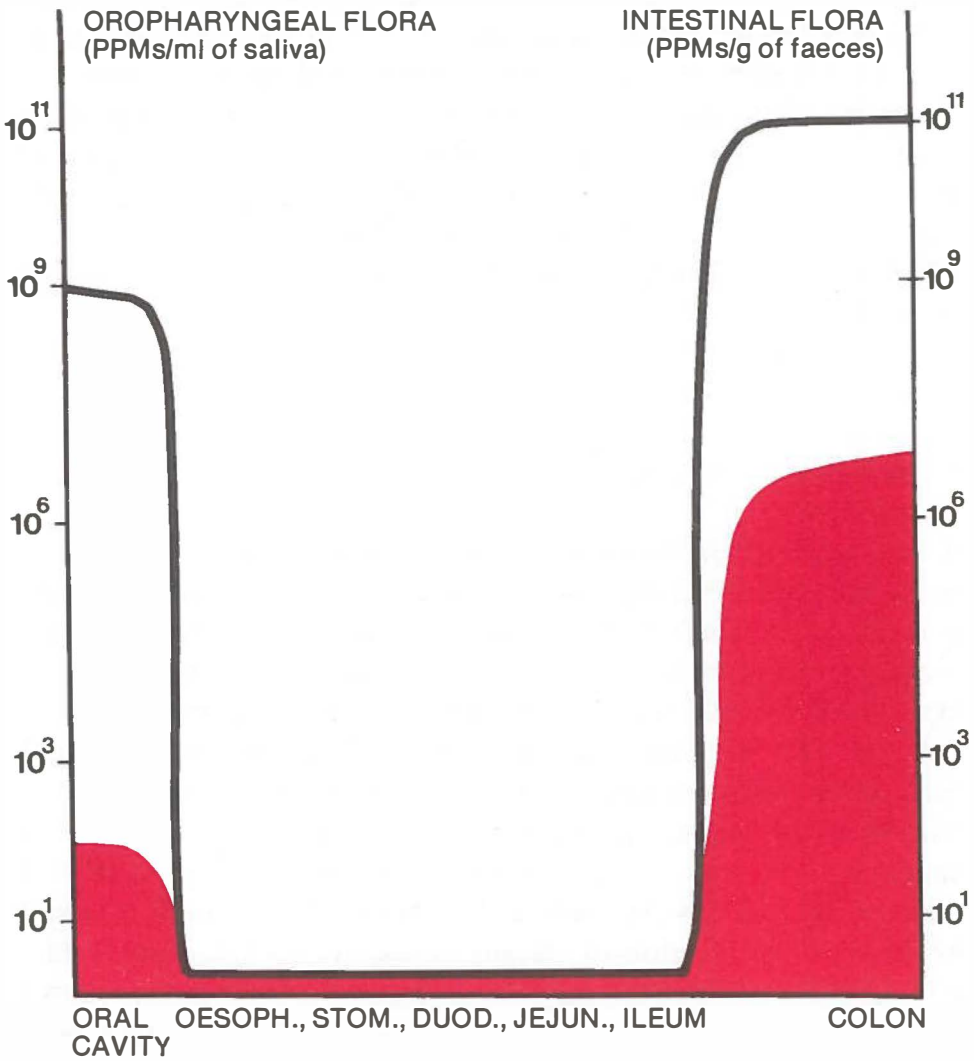


FIGURE 1.3
Colonization pattern of digestive tract under normal circumstances. High concentrations of (colonization resistance supporting) anaerobes in oropharynx and colon (black line) controlling the aerobic *Enterobacteriaceae* spp (red area).

Colonization resistance

Colonization resistance or CR is defined as the resistance of an individual (or of one of the individuals tracts) against colonization by aerobic potentially pathogenic microorganims. CR is a defense concept and an individuals characteristic. An intact and normal CR results in the colonization pattern (qualitative and quantitative) represented as mentioned in Figure 1-3[2]. CR of the digestive tract includes a complex of defense factors which limit or even prevent colonization of the digestive tract by aerobic PPMs. PPMs supplied by food and beverages entering the digestive tract via the oropharynx will meet eliminating forces which counteract their attempts to colonize. The aim of this CR mechanism is control and/or elimination of these PPMs from the digestive tube. The CR of the DT depends on four basic eliminating forces:

a. mechanical cleansing;
b. production of saliva repectively mucin;
c. desquamation of mucosal cells;
d. secretory immunoglobulin A (s-IgA) interfering with bacterial adherence.

A fifth and very important factor in establishing CR of the DT is the anaerobic part of the digestive tract microflora. The anaerobes play a key role in the control of the concentration of the aerobic flora in oropharynx and intestines. A sufficient number of anaerobes of the kind which normely colonizes the digestive tract constitue — 'in concert' — with his host a good control of the aerobic potentially pathogenic microorganisms. This mathematically results into a number of 10^5 aerobes *(Escherichia coli)* to 10^{10}anaerobes. How the anaerobes do realize this controlling function is not yet fully understood. It is however certain that they do it in two ways: *directly* by means of production of certain substances such as volatile fatty acids and bacteriocines to interfere in a direct way with the entering aerobes trying to colonize the digestive tube. But more important is the *indirect* contribution of anaerobes to the CR by means of stimulation of certain CR related host activities such as intestinal peristalsis, production of mucin and epithelial desquamation.

1. CR of the oropharyngeal cavity

Essential factors in the colonization resistance of the oropharynx are the integrity of the oral mucosa, mechanical cleansing by muscular actions of the tongue, cheeks and lips and by swallowing. This is greatly aided by saliva flow which in addition to lubricating the movements during chewing and swallowing makes it possible to swallow PPMs into the stomach. Desquamation of epithelial cells, the antimicrobial components in saliva (lysozyme, peroxidase, lactoferrin, complement etc.) as well as the indigenous oral flora (anaerobes, viridans streptococci) constitute a powerful defense system contributing to the oropharyngeal CR. The oral mucosa continuously bathing in saliva is covered by s-IgA the most important immunoglobulin secreted with saliva.

2. CR of the intestinal tract

On the analogy of the oropharynx mechanical cleansing at intestinal level is ensued by peristalsis and mucin has the same lubricating function as saliva in the oral cavity. Desquamation of mucosal cells to which bacteria adhere, s-IgA interfering with bacterial adherence and the anaerobic part of the microflora by its direct and indirect action against aerobes, all are involved in the control and/or elimination of aerobic PPMs *(Enterobacteriaceae)*.

The digestive tube stretching from the oropharynx to the anus forms one organ or entity also concerning the physiological CR factor. The colonization pattern of the oropharynx seems also to be controlled indirectly by intestinal anaerobes in the colon. A greater deal of the control of Gram-negative colonization in the oral cavity is perhaps not the result of direct interference but more an enhancing influence of colonic bacteria on the 'four major basic forces' of the host. This could imply that the digestive tract with its complex microflora can be regarded as an *organ* in which bacteria do not only participate in digestion of proteins and carbohydrates or in deconjugation of bile acids.

(II)
LOCAL IMMUNITY OF THE DIGESTIVE TRACT

The whole of the digestive tube stretching from mouth to anus is lined by epithelium in close contact with a strongly developed lymphoid system. This 'lymphoid organ' controlling entry of food an microbial substances and preventing invasion is called *gut associated lymphoid tissue (GALT)*[3, 4].

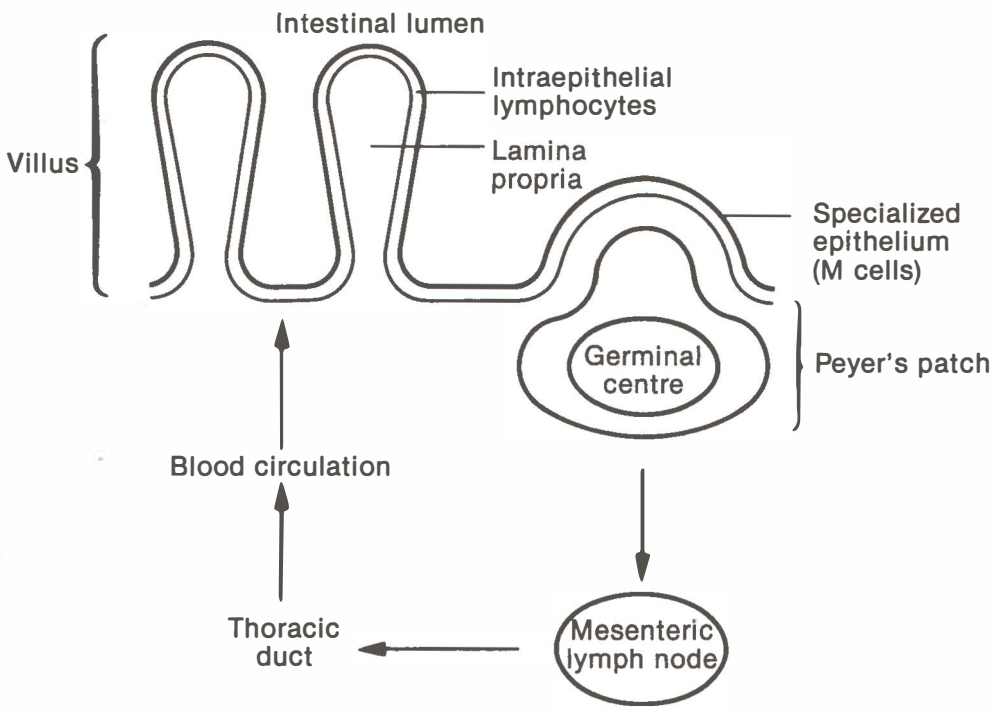


FIGURE 1.4
Antigenic sensitization in gut associated lymphoid tissue (GALT) (Peyer's patches), traffic of lymphoid cells from Peyer's patches to mesenteric lymph nodes, circulation and homing.

Anatomy

GALT consists of tonsils, Peyer's patches and appendix. The Peyer's patch is a nodule of lymphoid tissue consisting of a germinal centre lying in close apposition to a specialized epithelium containing a typical cell type, the microfold or M cell. These specialized M cells transport antigenic material from the intestinal lumen to underlying lymphoid cells (Figure 1.4). The antigen interacts with T- and B- small lymphocytes in what is essentially a traffic area. These cells are IgA precursor cells but do not produce IgA locally. The sensitized cells then migrate to the mesenteric lymph nodes undergoing a process of differentiation. Thereafter they enter the thoracic duct and to the gut mucosa where over 90% of lymphoid cells in the lamina propria are IgA secreting plasma cells (Figure 1.5).

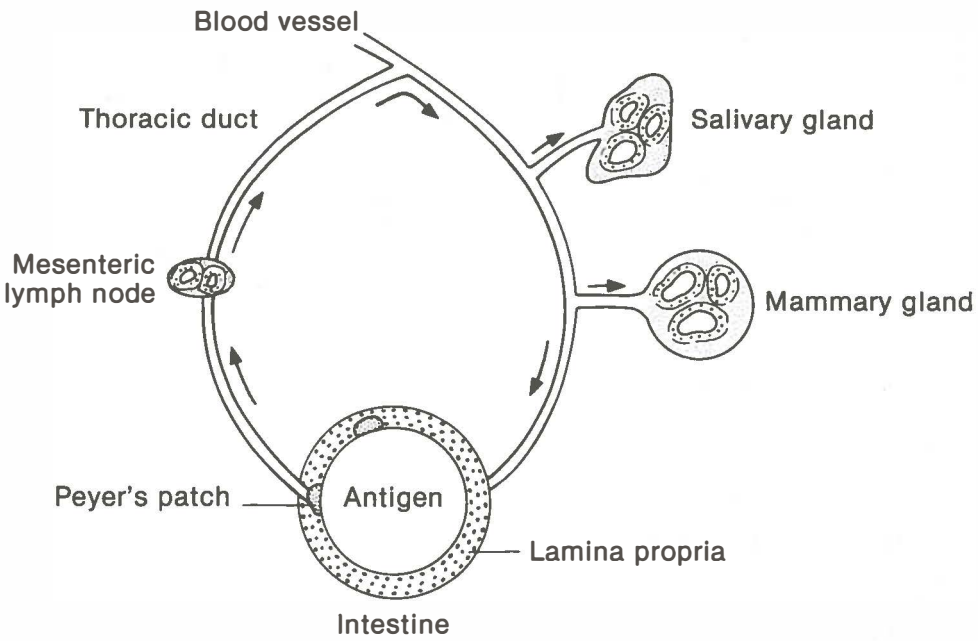


FIGURE 1.5
Selectively homing of IgA cells into salivary gland and intestines.

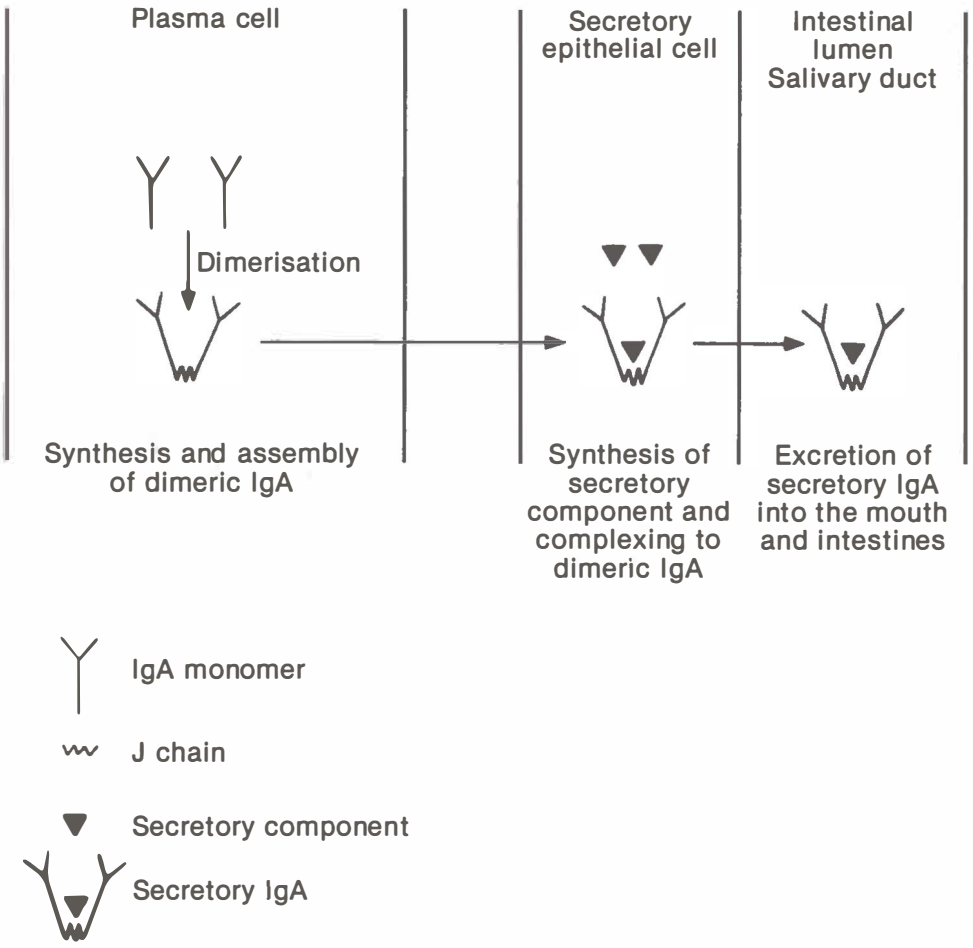


FIGURE 1.6
Synthesis, assembly and secretion of secretory immunoglobulin A.

Local immune response

Two molecules of IgA are combined by means of a joining (J) chain which is also secreted by local plasma cells. Dimeric IgA is then complexed by the secretory component, synthesized by the epithelial cells of salivary acini and intestinal mucosa; and the assembled secretory IgA or s-IgA is then transported into the duct lumen and excreted into the mouth and into the intestinal lumen of the gut (Figure 1.6). The advantage of s-IgA is that it is more resistant to proteolytic degradation than other immunoglobulins.

Function of s-IgA

The function of s-IgA has been described as an 'antiseptic paint' for mucosal surfaces. The current view of the mechanism of action of s-IgA is that the antibodies may combine with bacteria by 'coating' the microorganisms and so prevent their adherence to the corresponding receptors on the mucosal surface. This may prevent absorption of the vast array of food and bacterial antigens from the digestive tract and thereby prevent both overloading the systemic immune system and development of undesirable allergic responses.

The anaerobic microflora not only constitutes colonization resistance in close cooperation with the host, but also the local immune appartus contributes in an important way to the CR. Animal work shows that the anaerobes are never coated by s-IgA resulting in extreme high concentrations of $\geqslant 10^{10}$anaerobes per gram of faeces. The aerobes *(Enterobacteriaceae, Str. faecalis* and yeasts*)* are selectively coated by the local immune system as parts of the control mechanisms. An intact CR (anaerobes + s-IgA) results in numbers of 10^5 aerobes per gram of faeces. In this monograph the term colonization resistance comprehends colonization pattern of the digestive tract. The locale immune system (s-IgA) is treated separately.

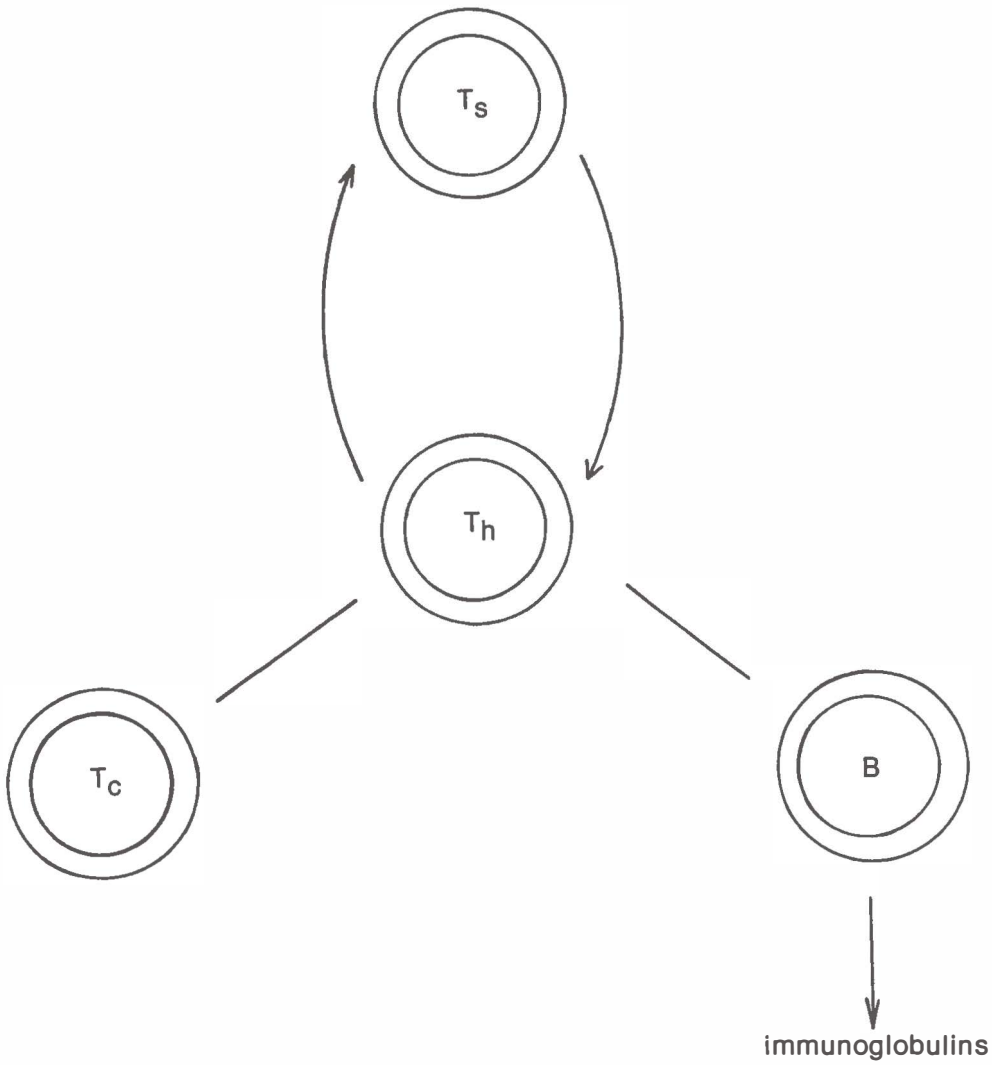


FIGURE 1.7
T-cell circuit sustaining immune homeostasis.
(T_s: T-suppressor cell; T_h: T-helper cell; T_c: T-cytotoxic cell; B: B-cell producing immunoglobulins).

(III)
SYSTEMATIC IMMUNE SYSTEM
(IMMUNE RESPONSE REGULATION)

In some circumstances when the local immune system is bypassed and overloaded some digestive tract-associated antigens enter into the circulation. This antigenic stimulation may result in the induction of systemic tolerance or systemic immunization (circulating antibodies)[5].

Systemic tolerance

Many data indicate that the local immune response play an important role in the induction of tolerance by oral route. A local immune response may result not only in a strong s-IgA response but also in the production of low titres of circulating IgA class antibody and the formation of IgA antibody-antigen complexes which, if formed in antigen excess, would be powerful tolerogens, i.e., the formation of IgA-containing immune complexes may reduce the absorption of further antigen and block an ensuing (IgM, IgG) systemic response.

The liver may also be important in the phenomenon of tolerance induction, although the mechanism by which it contributes is unknown.

Systemic immunization

Although the major emphasis has been on the protective role of the local immune system, in many cases a systemic response can be demonstrated. After intravenous immunization the majority of antibody production (IgM, IgG) occurs in the spleen. Digestive tract-associated antigens which have passed the mucosal barrier and entered the blood circulation are functionally equivalent to an intravenous injection of antigens and thus the major site of antibody production will be the spleen. The outcome of antigen triggering is governed by the balance between helper (inducer) T-cells and suppressor T-cells (Figure 1.7). These two discrete subsets of T-cells are critical for immune homeostasis. The inducer subset is central for the activation of B-cells (producing immunoglobulins as IgM or IgG), other T-cells, macrophages as well as for the hematopoietic differentiation. This

helper or inductive influence is regulated by the presence of suppressor T-cells that function to inactivate the inducer subset. The immune response on digestive tract-associated antigens is controlled by T-cell subpopulations with the aim of preventing sensitization against own digestive tract-associated antigens.

The liver also plays an important role in preventing production of circulating (precipitating IgM) antibodies induced by digestive tract-associated antigens. Intestinally derived antigens which have passed the local immune defences arrive via the portal circulation into the liver. The liver which is an important phagocytic organ renders the antigens non-immunogenic.

These three major (physiological) factors, the colonization resistance, local immune system and systemic immune system constitue the homeostasis by which an individual is capable to control his own endogenous flora.

REFERENCES

[1] *Gastrointestinal disease.* Eds. Sleisenger, M.H., Fordtran, J.S. Saunders Company, Philadelphia, 1978.

[2] *New criteria for antimicrobial therapy: maintenance of digestive tract colonization resistance.* Eds. van der Waaij, D., Verhoef, J. Excerpta Medica, Amsterdam, 1979.

[3] *Immunology of the gut.* Ciba Foundation Symposium. Excerpta Medica, Amsterdam, 1977.

[4] *Immunology of the gastrointestinal tract.* Eds. Asquith, P., Gell, P.G.H. Churchill Livingstone, Edinburgh, 1979.

[5] *Clinical gastrointestinal immunology.* Eds. Thomas, H.C., Jewell, D.P. Blackwell, Oxford, 1979.

CHAPTER TWO

OUTLINE OF THE INVESTIGATION INTO THE AETIOLOGY AND PATHOGENESIS OF INFLAMMATORY BOWEL DISEASES

„perhaps future studies should include also the concept of vulnerability of the host"

Kirsner, J.B.

The aim of this investigation is to study which part of the colonising (indigenous) flora (aerobes or anaerobes) can play a role in the aetiology and pathogenesis of inflammatory bowel diseases. Our investigation is merely based on previous work of the group of Shorter[1,2]. Shorter developed a working hypothesis for the aetiology and pathogenesis of inflammatory bowel diseases. His speculation was that 'inflammatory bowel disease results from the establishment of a state of hypersensitivity to antigen(s) of bacteria normally present in the individual's gastrointestinal tract. This state of hypersensitivity could develop as a result of increased permeability of the gut mucosa, allowing passage of nonenteropathic *Escherichia coli*. This increased permeability could occur in genetically predisposed individuals'.

Inflammatory bowel diseases consequently could be regarded as expression forms of immune reactivity ('hypersensitivity reaction') to endogenous intestinal bacterial antigens. In (genetically?) predisposed individuals with a decreased function of gut associated lymphoid tissue (GALT), translocation of bacterial antigens may occur and result in stimulation of the central immune apparatus (e.g., mesenteric lymph nodes, spleen). Endogenous intestinal bacteria sharing antigens with host constituents may induce cross-reacting antibodies, i.e., an-

tibodies which also complex with host tissue components. Finally, such cross-reacting antibodies are known to mediate in immunological inflammation reactions (type 2 or 3) and/or granuloma.

The intestinal antigens focused on in this investigation are selected on the basis of Shorter's hypothesis, i.e., the aerobic *Enterobacteriaceae*. Further support for this choice is provided by the following reasons:

1. The most likely candidates for increased translocation from the digestive tract lumen into the lymphoid organs seem to be the aerobic *Enterobacteriaceae* spp *(Escherichia coli, Klebsiella* spp, etc.*)*. The aerobic *Enterobacteriaceae* spp are *more invasive* than the anaerobes, although anaerobes outnumber the aerobes 10^4 times[3,4]. The anaerobes constantly present in high concentrations ($\geqslant 10^{10}$ per gram of faeces) form a kind of 'wall paper' of the intestinal mucosa; the aerobic *Enterobacteriaceae* normally found in lower concentrations ($<10^6$ per gram of faeces) may form only spots in that general anaerobic 'wall paper'. Under colonization resistance lowering conditions, the anaerobic part of the microflora decreases. This is generally immediately followed by a subsequent increase of the intestinal concentration(s) of aerobic *Enterobacteriaceae* spp. The latter then form no longer only small patches in the wall paper and may fill larger areas left open by the decimated anaerobic part. Under CR lowering conditions the aerobic *Enterobacteriaceae* spp are present in high numbers in the intestines; not only in its lumen but also in intimate relation with the intestinal mucosa resulting in an enhanced translocation.

2. A second reason why we focus our investigation on the aerobic *Enterobacteriaceae* spp is the evidence that the GALT system responds in general exclusively to the aerobic part of the digestive tract flora: potentially pathogenic bacteria such as aerobic *Enterobacteriaceae* spp, when they colonize in sufficiently high concentrations ($\geqslant 10^5$ per gram of faeces) are coated with secretory immunoglobulin A (s-IgA)[5,6]. The outnumbering anaerobes, however, have been found uncoated with s-IgA in mice. This suggests that either there is an immunological tolerance in the GALT for anaerobes or they do not penetrate the mucosal lining. Local immunological tolerance may be

caused by T-suppressor cells in the GALT. The function of such tolerance could be prevention of the production of s-IgA. s-IgA to anaerobes may limit their adherence and therewith their protective 'wall paper' function in the intestines. In addition, a state of tolerance may ensue prevention of development of inflammatory (tissue damaging) reactions to otherwise beneficial (CR constituting) bacteria or absorbed cell wall components of them[7,8].

3. The third reason why the scope of this investigation concerns the aerobic *Enterobacteriaceae* is the evidence of cross-antigenicity between *Enterobacteriaceae* and host constituents[9,10]. However, there is also evidence for the presence of cross-reacting antigens between antigens on the anaerobes and host tissue or organ specific constituents. Cross-reacting antibodies induced by anaerobes have also been reported[11,12].

4. The fourth reason why we place the study of *Enterobacteriaceae* colonization and host reactivity central, is formed by certain animal experiments. In mice, suggestive evidence has been described for a relation between the development of graft-versus-host disease and the absence of s-IgA secretion following conditioning for bone marrow engraftment and IgA-coated *Enterobacteriaceae* spp in faeces[5]. Furthermore, in guinea pigs, the development of ulcerative disease of the large intestine (induced by oral application with 5% degraded carrageenan) largely correlates with the presence of *Enterobacteriaceae* spp in the intestinal tract[13]. Because the ulcerative lesions in graft-versus-host disease are morphologically very similar to (chronic) IBD lesions, it seems indicated to investigate the incidence of s-IgA coated *Enterobacteriaceae* spp in IBD patients and compare it with the IgA coating incidence in normal healthy controls. Other investigators, however, have found that antimicrobial agents against obligate anaerobic bacteria could prevent the development of carrageenan induced caecal ulcerative lesions in hamsters. This suggests that the anaerobic part of the microflora may play a key role in the pathogenesis of experimental 'ulcerative colitis'[14,15,16].

How does the state of hypersensitivity become established? A consequence of translocation of enterobacterial antigens?

Translocation is defined as the passage of bacteria or bacterial antigens from the digestive tract through the epithelial mucosa into the lymphoid organs, e.g., mesenteric lymph nodes and spleen[17]. This penetration can result into stimulation of the systemic immune system with subsequent circulating antibodies[18]. Circulating (complement fixing) antibodies against endogenous digestive tract mucosa-associated flora may constitute a potentially harmful hypersensitivity state. Continuously released antigens, when they pass the mucosal lining may cause focal or more generalized inflammatory reactions in the submucosa along that part of the intestinal lining colonized (wall paper) by microorganisms to which the antibodies are directed. Cross-antigenicity between bacterial and (sub) mucosal antigens if it exists, could also cause massive tissue destruction by means of complement fixing antibodies interacting with bacteria and/or endogenous tissue antigens[19]. Prerequisites for translocation of indigenous bacteria from the digestive tract lumen into the lymphoid organs are:

(i) High concentrations of (aerobic) intestinal bacteria per gram of faeces ($\geqslant 10^6$ bacteria per gram of faeces). If *Enterobacteriaceae* spp candidate for translocation, high concentrations are to be expected in the faeces. This implies that the colonization resistance must be lowered.

(ii) Gut associated lymphoid tissue (GALT) function — associated with control of adherence of indigenous (aerobic) bacteria inside the lumen — should be decreased.

(iii) Translocation of intestinal bacteria or their antigens can be investigated by measuring the response of the systemic immune system to translocation, i.e., detection of increased titres of circulating antibodies against (translocating) bacteria or bacterial antigens.

(I)
IS COLONIZATION RESISTANCE OF DIGESTIVE TRACT OF IBD PATIENTS INTACT?

The colonization resistance (CR) of the digestive tract — as is discussed in Chapter one — is depending on eliminating forces of the host organism and on the anaerobic part of the microflora. A well functioning CR maintains the oropharyngeal flora 'normal', i.e., free of colonizing *Enterobacteriaceae* spp and into a faecal concentration of $< 10^6$ *Enterobacteriaceae* spp per gram of faeces. Many factors can interfere with this combined host and microflora associated defense mechanisms of the CR, e.g., antimicrobial agents (AMAs), operations, intubations, stress, etc. Antimicrobial drugs active against anaerobes *(Clostridia?)* and excreted in the digestive tract (via saliva and/or bile) affect the CR. Well known AMAs such as penicillin G, ampicillin, cefalothin, kanamycin, tetracyclines, etc., may decrease the CR to an important degree[20,21]. The possible impact of this negative influence on the digestive tract CR is 'overgrowth' by *Enterobacteriaceae* spp (resistant to the drugs used). Conditions of 'overgrowth' often involve an oropharyngeal concentration of $\geqslant 10^6$ resistant *Enterobacteriaceae* spp per ml of saliva and an intestinal concentration of $\geqslant 10^6$ resistant *Enterobacteriaceae* bacteria per gram of faeces (Figure 2.1). Similar shifts have been described following operations, intubations, stress, etc. these 'overgrowth' conditions are often found associated with translocation through the mucosa by those bacteria which are found in high concentrations.

Only under circumstances of decreased CR high concentrations of *Enterobacteriaceae* spp may be present in the digestive tract potentially followed by translocation into the lymphoid organs. Particularly, antimicrobial treatment of individuals and experimental animals with strongly CR decreasing AMAs has been observed to promote overgrowth, translocation and as a result subsequent sensitization. If translocation/sensitization occurs in the digestive tract, the most probable predeliction site for sensitization are the lymphatic organs associated with the oropharyngeal cavity. Although these lymph nodes may rapidly respond with the production of neutralizing an-

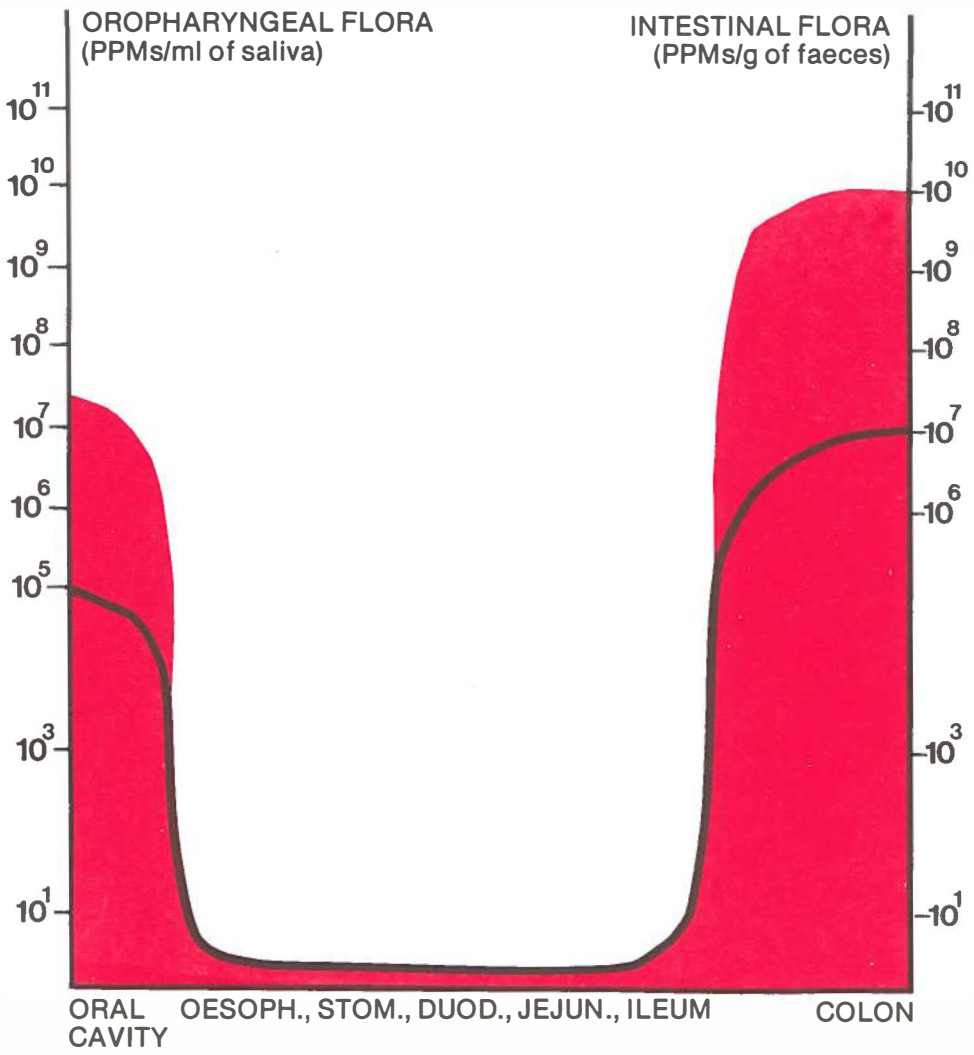


FIGURE 2.1
Colonization pattern of digestive tract in colonization resistance (CR) lowering conditions. A decrease of (CR constituting) anaerobes (black line) results in high concentrations of *Enterobacteriaceae* spp in oropharynx and intestines (red area).

tibodies to antigens, the clearance of penetrating antigens from the circulation, may be more delayed than those from the gut. In the latter situation the liver PMN-system drains intestinal antigens perhaps faster and more efficiently.

A classic example for demonstrating the oropharynx as possible site of translocation/sensitization are the post-streptococcal autoimmune diseases. Streptococcal angina is associated with high concentrations of *Streptococcus pyogenes* in the oropharyngeal cavity leading to translocation and sensitization. If it concerns certain serotypes the resulting circulating antibodies may cause in some patients (< 5 %) immunological inflammatory reactions in the heart, joints or kidneys[22,23].

The first stage of this investigation concerns the colonization pattern of aerobic bacteria in the digestive tract (oropharynx + intestines) in order to investigate the quantity of potentially pathogenic *Enterobacteriaceae* spp in the digestive tract and whether they are 'candidating' for translocation through the epithelium.

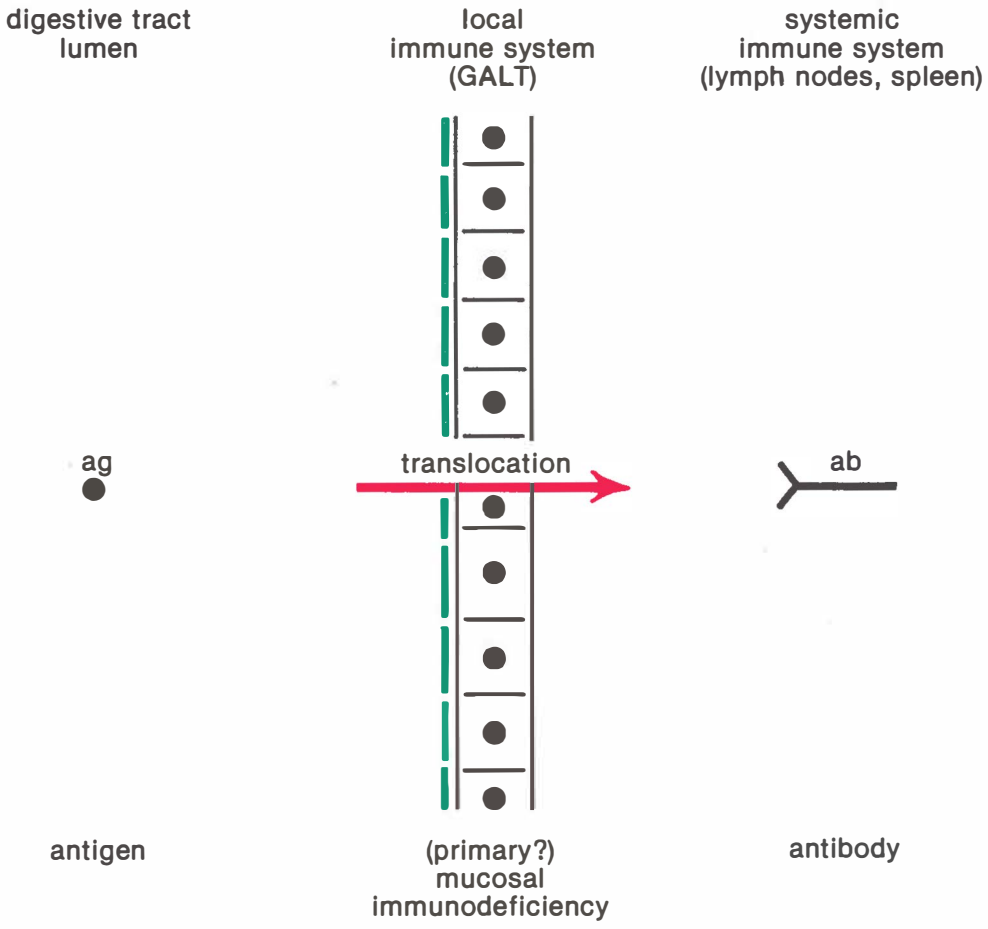


FIGURE 2.2
Enterobacterial antigen translocation in mucosal immunodeficiency (with subsequent antibody rise).

(II)
IS LOCAL IMMUNITY OF DIGESTIVE TRACT IN IBD PATIENTS DEFICIENT?

A function of the gut associated lymphoid tissue may be limiting of the adherence of *Enterobacteriaceae* spp to the mucosal cells. This mucosal-associated immune system may represent an important barrier to translocating of bacteria and subsequent systemic sensitization. A mechanism of action of local immunity is coating of most aerobic bacteria with secretory immunoglobulin A (s-IgA). This would limit their adherence to the mucosa[24,25].

In the literature there is increasing evidence for the possibility that a (primary?) mucosal immunodeficiency in the digestive tract of patients with inflammatory bowel disease plays a key role in the pathogenesis of their disease[26,27]. A dysfunction of GALT may facilitate translocation and possible subsequent sensitization[28] (Figure 2.2).

The second stage of this investigation includes the development of a technique for the evaluation of the function of the local immune system. The technique depends on the determination of the percentage of s-IgA coated aerobic *Enterobacteriaceae* spp in faeces.

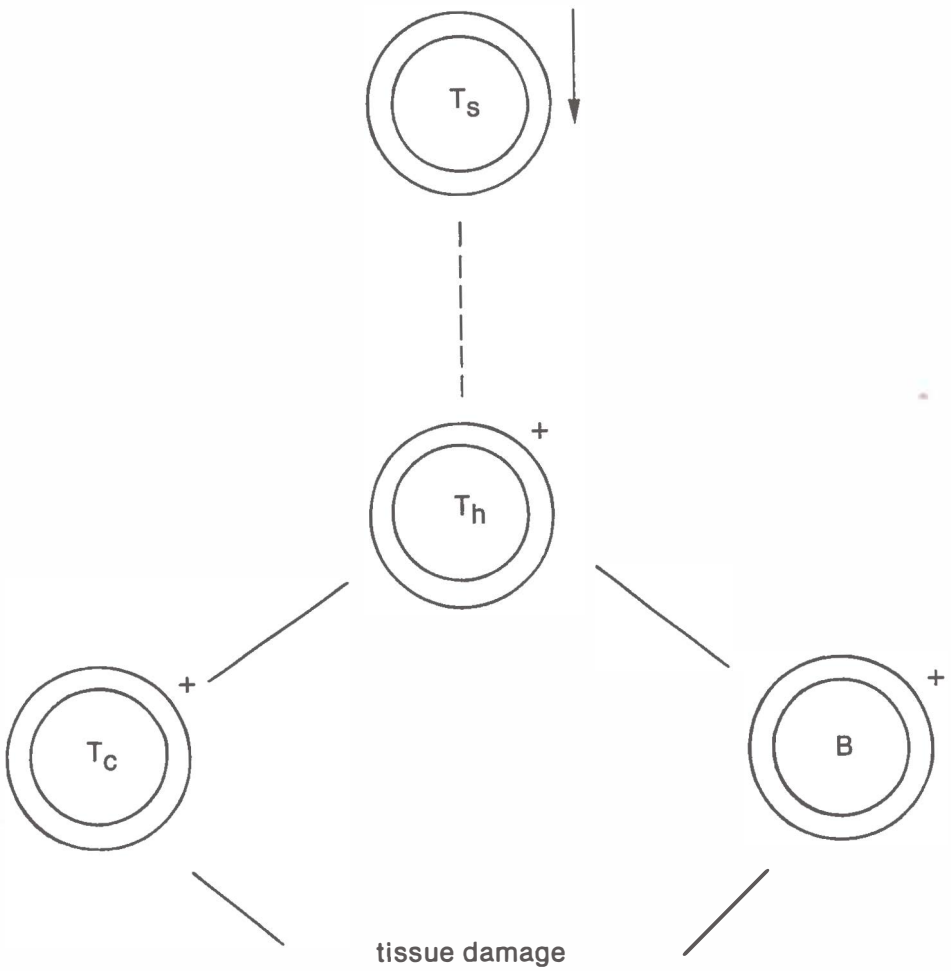


FIGURE 2.3
Scheme for possible defective immunoregulation in inflammatory bowel disease patients. (T_s: T-suppressor cell; T_h: T-helper cell; T_c: T-cytotoxic cell; B: B-cell producing immunoglobulins).

(III)
DOES THE SYSTEMIC IMMUNE SYSTEM OF IBD PATIENTS REACT TO (TRANSLOCATED) ENTEROBACTERIAL ANTIGENS?

The aim of this third stage of this investigation is the determination of circulating antibody concentrations to potentially pathogenic aerobic (translocated) enterobacterial antigens. In combination with the observations from stage (i) and (ii), information could be obtained, albeit in an indirect way, about the immunoregulatory homeostasis and perhaps more in general about the T-cell function. For, the outcome of translocation of bacteria and possible subsequent sensitization is — as discussed in Chapter one — governed by the balance between helper (inducer) T-cells and suppressor T-cells. The inducer subset activates B-cells producing IgM (IgG) antibodies. This helper or inductive influence is regulated by the presence of suppressor T-cells that function to inactivate the inducer subset. Recently, observations about a dysbalance between helper and suppressor T-cells in IBD patients are reported (Figure 2.3)[29,30]. A combination of a (primary?) mucosal immunodeficiency with a decrease or loss of T-suppressor cells probably provides a persistent production of circulating (complement fixing) antibodies to mucosa associated flora, maintaining in this way the state of hypersensitivity to these bacteria or components of these bacteria.

Patient selection. IBD patients who were admitted to the gastroenterology department and who had not been treated by AMAs and immunosuppressive therapy were selected. As controls acted healthy coworkers under identical conditions.
Oropharyngeal swabs and faecal portions were collected twice weekly; serum once a week.

In conclusion: to examine whether translocation of intestinal bacterial antigens from the digestive tract into the lymphoid organs is established by a combination of abnormalities, e.g., a mucosal immunodeficiency with a dysfunction of T-regulatory cells, we decided

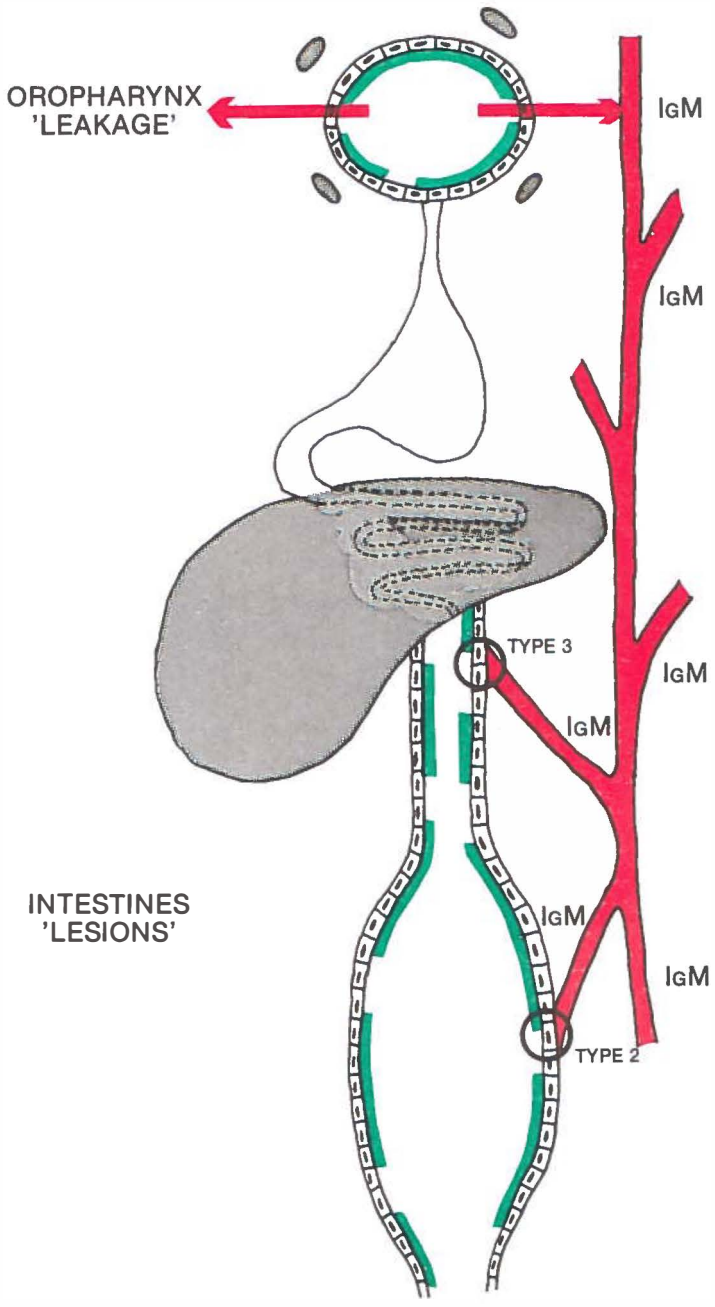


FIGURE 2.4
Diagram of a hypothetical model for the pathogenesis of inflammatory bowel diseases (IBDs).

to investigate: (i) the colonization pattern of aerobic *Enterobacteriaceae* spp (Chapter three), (ii) the local immunity (Chapter four) and (iii) the systemic immune system response to enterobacteria (Chapter five) in IBD patients and healthy control subjects. Whether *Enterobacteriaceae* spp are only good indices for these three study aims or whether they are involved in the pathogenesis of IBDs is discussed in Chapter six and Chapter seven.

Enterobacterial antigen translocation with subsequent hypersensitivity in mucosal immunodeficiency

Figure 2.4 shows the hypothetical model of this investigation into the aetiology and pathogenesis of inflammatory bowel diseases. This study includes three moments:

(i) *Digestive tract associated antigens.* As a consequence of decreased colonization resistance, high concentrations of aerobic *Enterobacteriaceae* spp can be present in the digestive tract lumen (oropharynx + intestines).

(ii) *Translocation: facilitated by a deficient gut associated lymphoid tissue.* Besides high concentrations of enterobacterial antigens a second condition necessary for translocation has to be fullfilled, i.e., a decreased function of GALT (green lining is broken). The most probable site of translocation may be the oropharyngeal cavity.

(iii) *State of hypersensitivity depending on the presence of circulating antibodies.* After translocation with subsequent sensitization at oropharyngeal level (red arrows), circulating antibodies (IgM class) may arise. These complement fixing antibodies mediate immune inflammatory reactions at intestinal level, i.e., the sites of high enterobacterial antigen supply. Two types of hypersensitivity reactions may be possible: type 2 reaction includes a cytotoxic (antibody-assisted lymphocyte mediated) mechanism, or, type 3 the (complex mediated) hypersensitivity reaction.

REFERENCES

[1] SHORTER, R.G., HUIZENGA, K.A., SPENCER, R.J. (1972). A working hypothesis for the etiology and pathogenesis of nonspecific inflammatory bowel disease. *Digestive diseases,* 17:1024-1032.

[2] SHORTER, R.G. (1974). Immunological aspects of gastrointestinal disease: an up-to-date account of inflammatory diseases such as ulcerative colitis and Crohn's disease. In: *Progress in Immunology* II. Eds. Brent, L., Holborow, J. North-Holland Publishing Company, Amsterdam, 4:209-219.

[3] VAN DER WAAIJ, D., TIELEMAN-SPELTIE, T.M., DE ROECK-HOUBEN, A.M.J. (1978). Relation between the faecal concentration of various potentially pathogenic microorganisms and infections in individuals (mice) with severely decreased resistance to infection. *Antonie van Leeuwenhoek,* 44:395-405.

[4] MYEROWITZ, R.L., MEDEIROS, A.A., O'BRIEN, T.F. (1971). Recent experience with bacillemia due to gram-negative organisms. *J.Inf.Dis.* 124:239-246.

[5] VAN DER WAAIJ, D., HEIDT, P.J. (1977). Intestinal bacterial ecology in relation to immunological factors and other defense mechanisms. In: *Food and Immunology*. Eds. Hambraeus, L., Hanson, L.A. Almqvist & Wiksell, Stockholm, 133-141.

[6] VAN SAENE, H.K.F., VAN DER WAAIJ, D. (1979). A novel technique for detecting s-IgA coated potentially pathogenic microorganisms in the human intestine. *J.Immunol.Methods,* 30:87-96.

[7] SWARBRICK, E.T., STOKES, C.R., SOOTHILL, J.F. (1979). Absorption of antigens after oral immunisation and the simultaneous induction of specific systemic tolerance. *Gut,* 20:121-125.

[8] LANCET (1979). Editorial. Gut reaction to antigen. *Lancet,* i:173.

[9] THAYER, W.R. Jr., BROWN, M., SANGREE, M.H., KATZ, M.D., HERSH, T. (1969). *Escherichia coli* 0:14 and colon hemagglutinating antibodies in inflammatory bowel disease. *Gastroenterology,* 57:311-318.

[10] ASHERSON, G.L., HOLBOROW, E.J. (1966). Autoantibody production in rabbits. VII. Autoantibodies to gut produced by the injection of bacteria. *Immunology,* 10:161-167.

[11] LYAMPERT, I.M., DANILOVA, T.A. (1975). Immunological phenomena associated with cross-reactive antigens of microorganisms and mammalian tissues. *Prog. Allergy,* 18:423-477.

[12] QUICK, J.D., GOLDBERG, H.S., SONNENWIRTH, A.C. (1972). Human antibody to *Bacteroidaceae. Am.J.Clin.Nutr.,* 25:1351-1356.

[13] VAN DER WAAIJ, D., COHEN, B.J., ANVER, M.R. (1974). Mitigation of experimental inflammatory bowel disease in guinea pigs by selective elimination of the aerobic Gram-negative intestinal microflora. *Gastroenterology,* 67:460-472.

[14] ONDERDONK, A.B., HERMOS, J.A., BARTLETT, J.G. (1977). The role of the intestinal microflora in experimental colitis. *Am.J.Clin.Nutr.,* 30:1819-1825.

[15] ONDERDONK, A.B., HERMOS, J.A., DZINK, J.L., BARTLETT, J.G. (1978). Protective effect of metronidazole in experimental ulcerative colitis. *Gastroenterology,* 74:521-526.

[16] ONDERDONK, A.B., BARTLETT, J.G. (1979). Bacteriological studies of experimental ulcerative colitis. *Am.J.Clin.Nutr.,* 32:258-265.

[17] BERG, R.D., GARLINGTON, A.W. (1979). Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. *Infect.Immun.,* 23:403-411.

[18] BERG, R.D. Ph.D. thesis.

[19] GOOD, R.A., RODEY, G.E. (1970). IgA deficiency, antigenic barriers, and autoimmunity. *Cell.Immunol.,* 1:147-148.

[20] VAN DER WAAIJ, D. (1979). Colonization resistance of the digestive tract as a major lead in the selection of antibiotics for therapy. In: *New criteria for antimicrobial therapy: maintenance of digestive tract colonization resistance.* Eds. van der Waaij, D., Verhoef, J. Excerpta Medica, Amsterdam, 271-282.

[21] VAN DER WAAIJ, D., BERGHUIS, J.M. (1974). Determination of the colonization resistance of the digestive tract of individual mice. *J.Hyg.,* 72:379-387.

[22] KAPLAN, M.H., MEYESERIAN, M. (1962). An immunological cross-reaction between group A streptococcal cells and human heart tissue. *Lancet,* i:706-710.

[23] KAPLAN, E.L., ANTHONY, B.F., CHAPMAN, S.S., AYOUB, E.M., WANNAMAKER, L.W. (1970). The influence of the site of infection on the immune response to group A streptococci. *J.Clin.Invest.,* 49:1405-1414.

[24] HEREMANS, J.F. (1975). The secretory immune system. A critical appraisal. In: *The immune system and infectious diseases.* Karger, Basel, 376-385.

[25] WILLIAMS, R.C., GIBBONS, R.J. (1972). Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. *Science,* 1977:697-699.

[26] ENGSTROM, J.F., ARVANITAKIS, C., SAGAWA, A., ABDOU, N.I. (1978). Secretory immunoglobulin deficiency in a family with inflammatory bowel disease. *Gastroenterology,* 74:747-751.

[27] GELZAYD, E.A., KRAFT, S.C., FITCH, F.W., KIRSNER, J.B. (1968). Distribution of immunoglobulins in human rectal mucosa. II. Ulcerative colitis and abnormal mucosal control subjects. *Gastroenterology,* 54:341-347.

[28] OWENS, W.E., BERG, R.D. (1980). Bacterial translocation from the gastrointestinal tract of athymic (nu/nu) mice. *Infect.Immun.,* 27:461-467.

[29] HODGSON, H.J.F., WANDS, J.R., ISSELBACHER, K.J. (1978). Decreased suppressor cell activity in inflammatory bowel disease. *Clin.Exp.Immunol.,* 32:451-458.

[30] GOODACRE, R., BIENENSTOCK, J. (1978). Loss of suppressor cell activity in intestinal lymphocytes from patients with Crohn's disease. *Gut,* 20:A910, abstract.

CHAPTER THREE

COLONIZATION RESISTANCE OF THE DIGESTIVE TRACT

,,la vie empêche la vie''

Pasteur, L.

I

OROPHARYNGEAL FLORA IN INFLAMMATORY BOWEL DISEASE PATIENTS

H.K.F. van Saene[1], W. Jansen[2], W.C. Veeger[3] and D. van der Waaij[4].

Laboratory for Medical Microbiology[1,4]
and Department of Gastroenterology[2,3],
University Hospital, Groningen, The Netherlands.

The oral flora of 25 inflammatory bowel disease patients (13 with Crohn's disease and 12 with ulcerative colitis) was compared with a control group of 25 healthy individuals. No significant differences were observed. The colonization resistance of the oropharynx of inflammatory bowel disease patients — which remains unimpaired — is discussed.

INTRODUCTION

The diseases known collectively as inflammatory bowel diseases (IBDs) are ulcerative colitis (UC) and Crohn's disease (CD). Their aetiopathogenesis is poorly understood. There is considerable evidence for the involvement of an auto-immunological proces[1]. One possibility is an altered immune reactivity to digestive tract-associated antigens, including enterobacterial antigens[2]. The site of sensitization could be the oropharynx similar to post-streptococcal auto-immunization which is observed only after infection of the throat and never following skin infection[3]. Once hypersensitivity is established, hyperimmune reactions (inflammation) can develop in the intestines. If the oral cavity is the 'porte d'entrée' for enterobacterial antigens

and if this oropharyngeal leakage leads to a state of hypersensitivity for these antigens, at least one condition should be present: (i) an abnormally high concentration of *Enterobacteriaceae* in the oropharynx, (ii) a constitutionally decreased colonization resistance (CR). The colonization resistance of the oropharynx is dependant on a multitude of factors such as integrity of mucosa, saliva flow, indigenous flora (viridans streptococci, anaerobes) together with secretory immunoglobulin A (s-IgA) in saliva[4,5]. An intact resistance to colonization results in an oral cavity free from *Enterobacteriaceae* as only a transient stay of Gram-negative bacilli in small numbers is tolerated by a well functioning oropharyngeal CR[6]. Many factors can interfere with the CR. Antimicrobial agents (AMAs) like penicillin, tetracyclin and chloramphenicol are known to induce a lowering of CR by affecting indigenous flora leading to emergence of (resistant) *Enterobacteriaceae* in the oropharynx[7,8,9]. Anaesthesia, operations, intubations etc. also lower CR by means of interfering with host organism factors like intact mucosa, saliva flow etc.[10,11]. In all circumstances *Enterobacteriaceae* can be detected in the oropharynx in high concentrations.

It is in the light of these considerations that we focus here on the oropharynx as possible site of sensitization.

SUBJECTS AND METHODS

Subjects

The subjects included in this study were 25 in-patients with a clinical diagnosis of IBD in the gastroenterology department of the University Hospital. They were selected when a prolonged hospital stay seemed likely and if they had not had AMAs for at least one month before hospitalization. When AMAs were used during the study, the patient was dropped from the study.

A group of 25 healthy volunteers, 10 laboratory co-workers (hospital-associated group) and 15 friends and neighbours of them (non-hospital associated group) served as a control group. No member of the control group had experienced any acute illness or

received any antimicrobial therapy in the preceding month nor during the 6 weeks of culturing.

Microbiological evaluation

The posterior pharynx of each subject was streaked with a cotton-tipped swab. Three agar plates were then corner streaked immediately with the swab, and its tip was broken off into broth. Cultures were done on sheep blood agar (Oxoid), MacConkey agar (Oxoid), yeast isolation agar (Merck) and in brain heart infusion (BHI) broth (Oxoid). All agar plates were incubated at 37° C; Mac Conkey agar plates were examined after one night, yeast isolation and sheep blood agar plates after two nights. The aerobic bacteria in all cultures were qualitatively and semi-quantitatively estimated. The normal indigenous oropharyngeal flora (viridans streptococci) were identified by colonial morphology and type of haemolysis on blood agar. The other bacteria (*St. aureus, Enterobacteriaceae, Pseudomonadaceae* and yeasts) were identified by means of standard bacteriological techniques[11]. Semi-quantitative estimation of all these bacteria was made on a scale of +1 to +5 according to their presence in broth (+1) and growth density in the four quadrants of the agar plates (+2 to +5). In this study oropharyngeal carriership was defined as the condition in which an identical bacterium was isolated from more than 60% of throat swab samples during the sample period. Survey of the number of subjects and cultures is presented in Table 1.

TABLE 1
Number of subjects and cultures

	Number of subjects	Number of cultures	Number of cultures/subjects
Normal subjects	25	250	10
- non-hospital associated	15	150	10
- hospital associated	10	100	10
IBD patients	25	295	11.8
- Crohn's Disease (CD)	13	154	11.8
- Colitis Ulcerosa (CU)	12	141	11.8

RESULTS

The prevalence of *St. aureus, Enterobacteriaceae, Pseudomonadaceae,* yeasts and viridans streptococci in oropharyngeal cultures on single-culture surveys is shown in Table 2. No significant differences were observed between the normal group and the IBD patients. With the exception of viridans streptococci, the total number of colonies of

TABLE 2
Percentage of oropharyngeal cultures containing

	Gram + cocci	Gram-bacilli		Yeasts	Str.
	St. aureus	*Enterobact./Pseudom.*		*C. albicans*	viridans
Normal subjects	8.8	10.0	0.8	29.2	100
- non-hospital associated	6.0	6.0	0.6	32.6	100
- hospital associated	13.0	16.0	1.0	24.0	100
IBD patients	22.7	12.8	—	17.3	100
- Crohn's Disease (CD)	18.8	12.3	—	18.8	100
- Colitis Ulcerosa (CU)	26.9	13.5	—	15.6	100

TABLE 3
Mean growth densities

	Gram + cocci	Gram-bacilli	Yeasts	Str.
	St. aureus	*Enterobact./Pseudom.*	*C. albicans*	viridans
Normal subjects	+1.7	+1.3	+1.6	+3.9
- non-hospital associated	+1.2	+1.3	+1.9	+4.0
- hospital associated	+2.0	+1.4	+1.1	+3.6
IBD patients	+1.7	+1.5	+1.8	+4.1
- Crohn's Disease (CD)	+2.1	+1.7	+2.0	+4.0
- Colitis Ulcerosa (CU)	+1.3	+1.2	+1.6	+4.3

all species observed on the agar plates was small. The mean growth densities varied from +1.3 to +1.8, i.e., potentially pathogenic microorganisms (PPMs) were grown only in broth medium and in the first quadrant (Table 3). The Gram-negative bacilli isolated are listed in Table 4. The absence of oropharyngeal cultures positive for *Proteus* spp and *Pseudomonas* spp in the hospitalized IBD group is noteworthy. One healthy subject turned out to be an *E.coli* carrier, and three of the IBD patients were carriers of *Klebsiella* spp and *Citrobacter* spp. The carriage rate of *St. aureus* however, differed: 4% (1/25) in the control group and 16% (4/25) in the patient group. *C. albicans*

TABLE 4
Gram-bacilli prevalence in oropharynx of

	Normal subjects	IBD patients
ENTEROBACTERIACEAE:		
Escherichia coli	11	2
Klebsiella spp		
- *Klebsiella pneumoniae*	—	6
- *Klebsiella oxytoca*	4	8
Enterobacter spp		
- *Enterobacter aerogenes*	1	1
- *Enterobacter cloacae*	3	10
- *Enterobacter agglomerans*	—	2
Serratia liquefaciens	3	1
Hafnia alveï	2	—
Citrobacter freundii	—	8
PSEUDOMONADACEAE:		
Pseudomonas aeruginosa	1	—
	25/250 (10%)	38/295 (12.8%)

carriership on the other hand was found to be more or less equal in both groups: 28% (7/25) in the healthy subjects and 12% (3/25) in the IBD group. The results of multiple-culture surveys are presented in Table 5.

TABLE 5
Oropharyngeal carriership

	Normal subjects	IBD patients
Gram + cocci		
St. aureus	1	5
Gram-bacilli		
Escherichia coli (5144552)	1	
Klebsiella oxytoca (5245773)		1
Klebsiella pneumoniae (5215773)		1
Citrobacter freundii (1204572)		1
Yeasts		
Candida albicans	7	3
Str. viridans	25	25

DISCUSSION

Essential factors in the colonization resistance (CR) of the oropharynx are the integrity of the oral mucosa, mechanical cleansing by muscular actions of the tongue, cheeks and lips and by swallowing. This is greatly aided by the saliva flow which, in additions to lubricating the movements during chewing and swallowing, makes it possible to clear the area and to swallow ingested PPMs into the stomach. The antimicrobial components in saliva (lysozyme, peroxidase, lactoferrin, complement etc.) as well as the indigenous oral flora (viridans streptococci) constitute a powerful defense system contributing to the oropharyngeal CR. The oral mucosa, continuously bathing in saliva, is covered by secretory immunoglobulin A (s-IgA) the most important immunoglobulin secreted with saliva. Secretory IgA may prevent absorption of bacterial antigens from the oropharynx and thereby prevent both overloading the local immune system and development of undesirable hypersensitive immune responses[13]. A mechanism of action of s-IgA which may be common to many PPMs is to prevent their adherence to (corresponding) receptors on the mucosal surface[14].

In our study the CR of the oropharynx has been measured in two ways. (i) Directly by monitoring the viridans streptococci: in IBD patients, viridans streptococci were constantly found in high numbers, indeed higher than in the healthy control group. The indigenous flora of the mouth which may contribute to the prevention of colonization, appeared therefore unaltered in our patients[15]. (ii) Indirectly by measuring *Enterobacteriaceae* spp, *St. aureus* and *C. albicans*. In the literature, the prevalence of Gram-negative bacilli in normal oropharyngeal flora varies from 2% to 18%[6,16]. We found a rate of 10% in the control group. In the IBD group the prevalence was 13%. In the healthy control group one *Escherichia coli* carrier was found while two IBD patients appeared colonized by *Klebsiella* and one patient by *Citrobacter*. The healthy *E. coli* colonized volunteer caught cold during the sampling period resulting in this case to altered oropharyngeal mucosa to which Gram-negative bacilli may adhere more easily[17]. The three IBD patients who contributed to about the

half of the oropharyngeal cultures with Gram-negative bacilli in this group, were quite ill during the study period. Johanson found that the appearance of Gram-negative flora in hospitalized patients correlated well with the clinical estimate of their degree of illness. Furthermore, that in severely ill patients the oropharyngeal mucosa is modified by the critical nutrition state leading to enhanced adherence by Gram-negative bacilli[18]. The numbers of positive *C. albicans* throat cultures did not show much difference between the healthy and the patient group. In fact in the controls there was evidence for a higher degree of carriership. Where perhaps a difference was manifest was a non-significant higher incidence of *St. aureus* in the IBD group. In this investigation only one control subject appeared carrier (4 %), in the IBD population 5 carriers were found (20 %). But in healthy subjects an incidence varying between 10 % and 40 % has been reported[19].

The conclusion that no significant differences were demonstrated in the number and types of PPMs present in the two groups suggests — indirectly — that the oropharyngeal resistance to colonization in IBD patients by ingested PPMs is intact and normal. The hypothesis postulated in the introduction that the oropharynx may be the site of sensitization for bacterial antigens which are involved in the pathogenesis of IBDs is not supported by our findings. We found neither a high concentration of *Enterobacteriaceae* spp nor a constitutionally decreased CR, in subjects where the inflammatory bowel disease was already established and active. If our hypothesis is valid, then the conditions of decreased CR may have existed only temporarily, i.e., only at the time of sensitization, e.g. a few weeks before the IBD onset. We note in this connection that Schachter and Kirsner[20] reported that upon careful questioning some patients recalled an earlier excessive intake of AMAs for respiratory tract or other infections (often penicillin taken orally). This suggests the possibility of an iatrogenic disturbance of digestive tract CR. In turn this may have permitted an anhanced 'take' and increased concentrations of (resistant) *Enterobacteriaceae* species in the oropharynx. Other patients reported operations (associated with intubations), emotional crises and periods of fatigue. In most instances no significant antecedent events were described.

ACKNOWLEDGEMENTS

We thank the nursing staff of the wards and the volunteers for their collaboration. We want to express our appreciation to Mrs. G. Helmus and Mrs. E. van Santen for technical assistance and Mrs. S. Swart for secretarial assistance.

REFERENCES

[1] JEWELL, D.P., HODGSON, H.J.F. (1976). Autoimmune and inflammatory diseases of the gastrointestinal tract. In: *Immunological aspects of the liver and gastrointestinal tract.* Eds. Ferguson, A., Mac Sween, R.N.M. Medical and Technical Press, Lancaster,203-250.

[2] SHORTER, R.G. (1974). Immunological aspects of gastrointestinal disease: an up-to-date account of inflammatory diseases such as ulcerative colitis and Crohn's disease. In: *Progress in Immunology* II. Eds. Brent, L., Holborow, J. North-Holland Publishing Company, Amsterdam,4:209-219.

[3] KAPLAN, E.L., ANTHONY, B.F., CHAPMAN, S.S., AYOUB, E.M., WANNAMAKER, L.W. (1970). The influence of the site of infection on the immune response to Group A streptococci. *J. Clin. Invest.*,49:1405-1414.

[4] LEHNER, T. (1980). Oral Immunity. In: *Immunology of oral diseases.* Eds. Roitt, I.M., Lehner, T. Blackwell, Oxford,297-322.

[5] VAN DER WAAIJ, D. (1981). Colonization resistance of the digestive tract with special emphasis on the oropharynx. In: *Developments in antibiotic treatment of respiratory tract infections.* Ed. R. van Furth. Martinus Nijhoff Publishers, The Hague, 53-67.

[6] ROSENTHAL, S., TAGER, I.B. (1975). Prevalence of Gram-negative rods in the normal pharyngeal flora. *Ann. Int. Med.,* 83:355-357.

[7] SMITH, J.W., BLOOMFIELD, A.L. (1948). The effect of penicillin on the aerobic bacterial flora of the normal throat. *Stanford Med. Bull.,* 6:469-480.

[8] MEADS, M., ROWE, W.P., HASLAM, N.M. (1951). Alterations in the bacterial flora of the throat during oral therapy with aureomycin. *Arch.Int.Med.*,87:533-540.

[9] McGOVERN, J.J., PARROTT, R.H., EMMONS, C.W., ROSS, S., BURKE, F.G., RICE, E.C. (1953). The effect of aureomycin and chloramphenicol on the fungal and bacterial flora of children. *New Engl.J.Med.*,248:397-403.

[10] DACK, G.M., PETRAN, E. (1934). Bacterial activity in different levels of the intestine. *J.Inf.Dis.*,54:204-220.

[11] REDMAN, L.R., LOCKEY, E. (1967). Colonisation of the upper respiratory tract with Gram-negative bacilli after operation, endotracheal intubation and prophylactic antibiotic therapy. *Anaesthesia*,22:220-227.

[12] MURRAY, P.R. (1978). Standardization of the Analytab Enteric (API 20E) system to increase the accuracy and the reproducibility of the test for biotype characterization of bacteria. *J.Clin.Microbiol.*, 8:46-49.

[13] STOKES, C.R., SOOTHILL, J.F., TURNER, M.W. (1975). Immune exclusion is a function of IgA. *Nature*, 255:745-746.

[14] WILLIAMS, R.C., GIBBONS, R.J. (1972). Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. *Science*, 177:697-699.

[15] SPRUNT, K., REDMAN, W. (1968). Evidence suggesting importance of role of interbacterial inhibition in maintaining balance of normal flora. *Ann.Int. Med.*, 68:579-590.

[16] CETIN, E.T., ANG, O., TORECI, K., BERKITEN, R. (1971). Investigations on aerobic oral and nasal flora of university students. *Path.Microbiol.*, 37:185-193.

[17] FAINSTEIN, V., MUSHER, D.M., CATE, T.R. (1980). Bacterial adherence to pharyngeal cells during viral infection. *J.Inf.Dis.*, 141:172-176.

[18] JOHANSON, W.G., PIERCE, A.K., SANFORD, J.P. (1969). Changing pharyngeal bacterial flora of hospitalized patients. *New Engl.J.Med.*, 281:1137-1140.

[19] MILLIAN, S.J., BALDWIN, J.N., RHEINS, M.S., WEISER, H.H. (1960). Studies on the incidence of coagulase positive staphylococci in a normal unconfined population. *Am.J.Public Health*, 50:791-798.

[20] SCHACHTER, H., KIRSNER, J.B. (1980). *Crohn's disease of the gastrointestinal tract.* Wiley & Sons, New York, 25-28.

II

INTESTINAL FLORA IN INFLAMMATORY BOWEL DISEASE PATIENTS

H.K.F. van Saene[1], Willy Jansen[2], W.C. Veeger[3], G.W. Welling[4] and D. van der Waaij[5].

Laboratory for Medical Microbiology[1,4,5] and Department of Gastroenterology[2,3], University Hospital, Groningen, The Netherlands.

Longitudinal qualitative and quantitative cultures of the aerobic faecal flora of 25 inflammatory bowel disease patients (13 with Crohn's disease and 12 with ulcerative colitis) and of 25 healthy adults (10 hospital-associated and 15 non-hospital-associated normal subjects) were carried out to compare the composition of the aerobic part of the intestinal microflora. As a measure of the anaerobic part of the intestinal flora, βaspartylglycine, a dipeptide appearing in cases of disturbed anaerobic flora, was determined in both groups. No significant differences were found between the patient and the control group: for both groups normal aerobic flora remained approximately constant in time (10^4-10^7 microorganisms per gram of faeces) and in both groups no β-aspartylglycine was detected indicating that also the anaerobic flora shows no major differences. The fact that the colonization resistance of the intestinal tract remains unimpaired in inflammatory bowel disease patients and possible reasons for this are discussed.

INTRODUCTION

Thirty years ago Seneca and Henderson reported normal intestinal flora in inflammatory bowel disease (IBD) patients (Seneca and Henderson, 1950). They were the first among many investigators to study bacterial flora from various levels of the gastrointestinal tract of IBD patients (Weinstein, 1961; Cooke, 1967; Gorbach et al., 1968; Vince et al., 1972). The most surprising finding in all these studies is the similarity between IBD patients and healthy controls: aerobic as well as anaerobic flora are very similar in both groups. Only slight increases of *Escherichia coli* and *Streptococcus faecalis* were noted (van der Wiel-Korstanje and Winkler, 1975; Keighley et al., 1978).

Studies in animals and humans by the group of van der Waaij reveals a delicate 'balance' in activity of normal intestinal microflora: the outnumbering anaerobes have been shown to control colonization of the intestines by aerobes (van der Waaij, 1971). Normally different species keep contaminating the intestines by oral route, usually colonizing the gut for short periods and in low concentrations (transient bacteria). Very few microorganisms can persist for several weeks or months (resident bacteria). Certain species of the anaerobic intestinal microflora function as a natural barrier against newly acquired microorganisms by mechanism as yet only partially understood. This barrier has been called colonization resistance (van der Waaij, 1979).

This resistance to colonization — due to the anaerobic microflora — can be directly measured by qualitative and quantitative culturing of anaerobes. Because anaerobic culturing and identification techniques are difficult, time-consuming and expensive, a test was developed to detect the dipeptide β-aspartylglycine, known to be an adequate indicator for the presence of the CR-responsible (physiologic) part of the anaerobic flora (Welling at al., 1980). Endogenous proteins and proteins from the diet are continously degraded to amino-acids by proteolytic enzymes from the host and the bacteria in the intestinal tract. When the anaerobic microflora is affected, β-aspartylglycine accumulates in the faeces. Its unusual peptide bond can only be cleaved by bacterial enzymes which would result in aspartic acid and glycine. β-aspartylglycine is absent in stools of in-

dividuals with a normal bowel flora. A second indirect way of investigating the completeness of the anaerobic flora is monitoring the aerobic colonization pattern of the stools (van der Waaij and Berghuis-de Vries, 1974). Low aerobic concentrations and short colonization periods of newly acquired aerobes imply that the anaerobic microflora are (remained) intact.

In view of the above, we have examined the faecal flora of 25 IBD patients (13 with Crohn's disease and 12 with ulcerative colitis) versus a control group of 25 normal adults in an attempt to assess the frequency and significance of differences arising.

SUBJECTS AND METHODS

Subjects

The 25 patients studied were adults for which prolonged hospitalization seemed likely at the time of their admission to the gastroenterology department of the University Hospital. No patient selected for the study had been given antimicrobial agents (AMAs) in the month prior to hospitalization nor during the 6 weeks of bacteriological monitoring.

A group of 25 healthy volunteers, 10 laboratory co-workers (hospital-associated group) and 15 of their friends and neighbours (non-hospital associated group) kindly served as a control group. None of the control people had experienced acute illness or received any antimicrobial therapy during the 6 weeks of study or in the month preceding it.

Studies on faeces

Specimens of faeces were obtained twice weekly for six consecutive weeks. Each faecal specimen was processed within 30 minutes of being passed.

Monitoring of aerobic flora

Concentrations of the aerobic gram-negative bacilli, yeasts and enterococci were determined as follows: one gram of faeces was

homogenized in 9 ml of brain heart infusion broth (Oxoid), serially diluted (1:10) and incubated for 18 hours at 37° C; thereafter, all dilutions showing bacterial growth were inoculated onto MacConkey agar (Oxoid), yeast isolation agar (Merck) and kanamycin aesculin azide ager (Oxoid). Concentrations of staphylococci in faecal samples were measured by a serial dilution in a salt containing liquid medium (10 % NaCl, Oxoid). After incubation all dilutions were subcultured on sheep blood agar (Oxoid). Identification and typing were performed by means of standard bacteriological techniques. All results were expressed as the $\log_{10}$ of the number of organisms per gram (wet weight) of faeces.

In this study we defined a carrier as one where *Staphylococcus aureus* and *Candida albicans* was cultured in more than 60 % of the faecal samples during the study period.

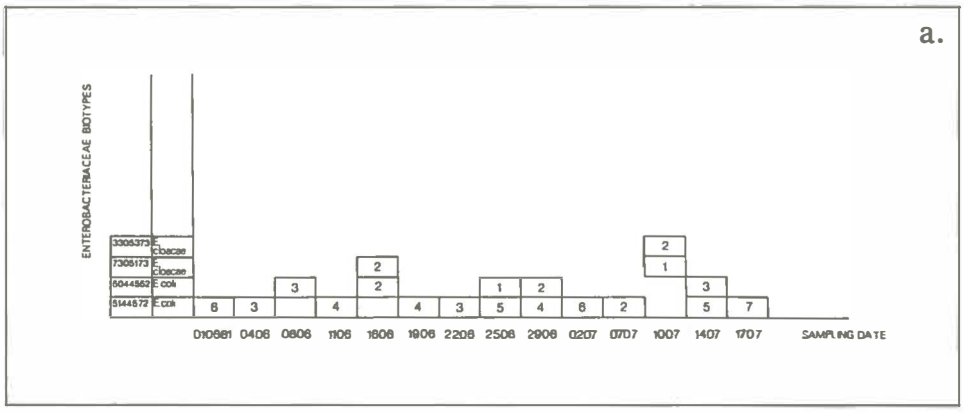
β-aspartylglycine as measure of the anaerobic faecal flora

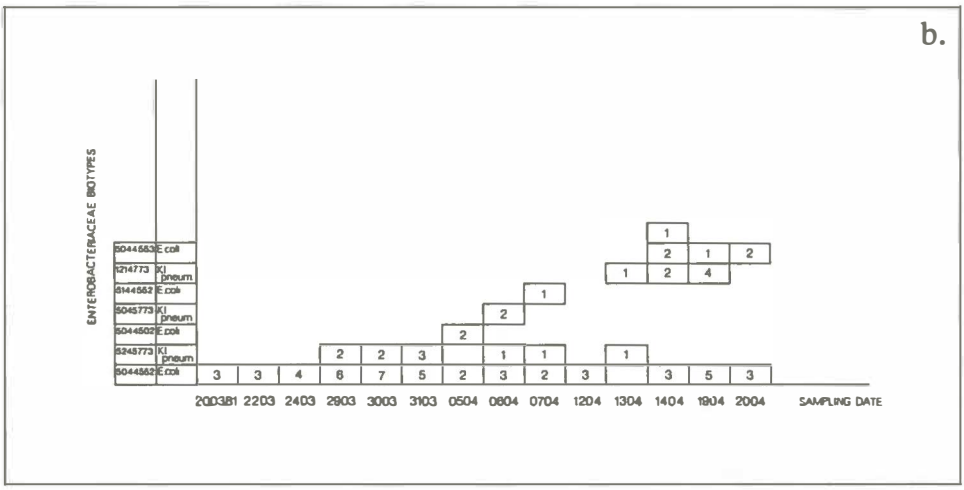
The techniques for β-aspartylglycine detection in faeces are previously described in detail (Welling and Groen, 1978). In brief, a 25 % w/v faecal suspension was centrifuged for 15 minutes at 15,000 rpm. 80 µl of the super natant was subjected to highvoltage paper electrophoresis at pH 3.5. After staining with ninhydrin and drying at 150° C the paper was examined for the presence of a clear blue spot of β-aspartylglycine.

RESULTS

Composition of the aerobic faecal microflora

Longitudinal (minimum 6 weeks) qualitative and quantitative culturing on 573 samples (11.4 samples per individual) was carried out. Figure 1 shows *Enterobacteriaceae* colonization patterns in normal controls (a,b) and in IBD patients (c,d). The flora of the IBD patients was similar to that of healthy subjects. The resident flora in both groups included *Escherichia coli* only. The transient flora consisted of *Klebsiella* spp, *Citrobacter* spp etc. *Salmonella, Proteus* or *Pseudomonas* spp were not detected. The four diagrams show that in





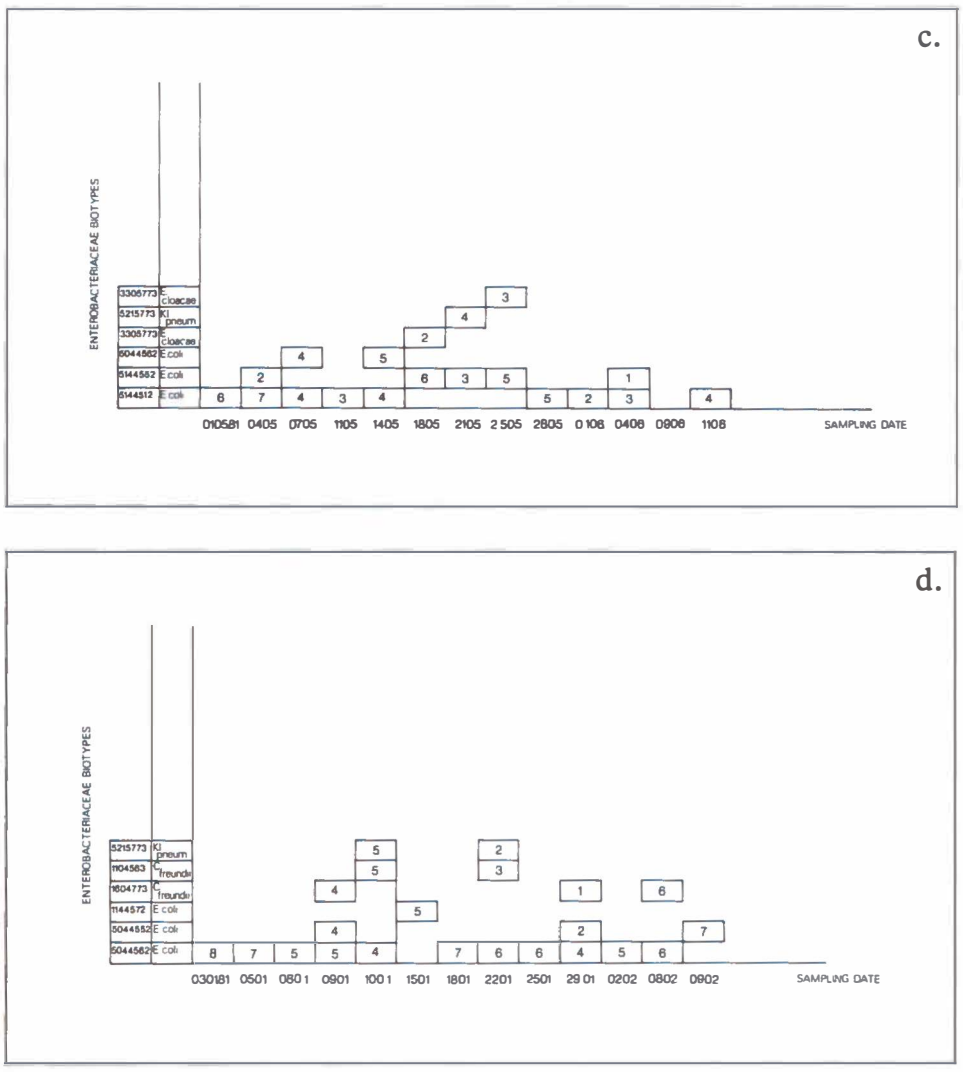


FIGURE 1

Digestive tract colonization by *Enterobacteriaceae*. Numbers in blocks give the concentration of *Enterobacteriaceae* expressed as the $\log_{10}$ of bacteria per gram of faeces. Colonization pattern of digestive tract of a non-hospital associated volunteer (a), of a hospital-associated volunteer (b), of a Crohn's disease patient (c) and of a colitis ulcerosa patient (d).

each subject the colonization resistance apparently remained intact: all newly acquired *Enterobacteriaceae* were eliminated from the gut; they were only able to colonize the intestines in low concentrations for short periods. No significant differences were demonstrated in the number of aerobic Gram-positive as well as Gram-negative bacteria present in the two groups (Figure 2). In both groups, IBD and control,

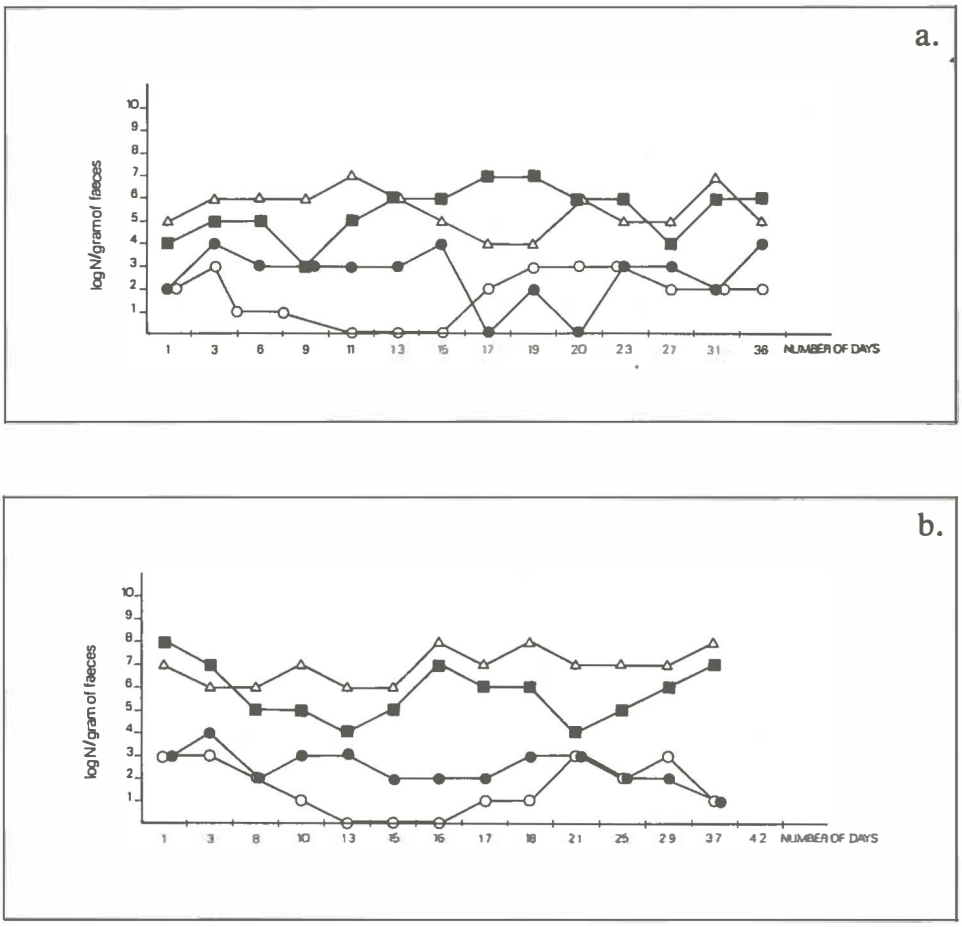


FIGURE 2
Aerobic faecal flora (■: *Enterobacteriaceae,* Δ: enterococci, ○: yeasts, ●: *St. aureus*): serial counts of stools of a normal subject (a) and of an IBD patient (b).

Enterobacteriaceae and enterococci remained approximately constant during the study period (10^4 - 10^7 bacteria per gram of faeces).

The carriage rate of *St. aureus* however differed, being 8 % (2/25) in the control group and 24 % (6/25) in the patient group. By contrast, the rate for *C. albicans* was 32 % (8/25) in the healthy subjects, and 16 % (4/25) in the IBD group. The concentrations of these two microorganisms in the faeces were slightly lower than the numbers of *Enterobacteriaceae* and enterococci (Figure 2).

Detection of β-aspartylglycine

No β-aspartylglycine could be found in the faecal specimens of the healthy group nor in those of the IBD group.

DISCUSSION

This study shows that the colonization resistance (CR) of the gut in inflammatory bowel disease (IBD) patients functions normally. It is well known that normal intestinal flora contributes significantly to a scale of physiologic processes (Gordon, 1960; Abrams et al., 1963; Donaldson, 1964, Abrams and Bishop, 1967). However, it is not generally appreciated that normal microbial flora of the intestines also constitutes one of the most important defence mechanisms to colonization and/or infection (Finger and Wood, 1955; Dubos and Schaedler, 1962; Bonhoff et al., 1964; Rosenthal, 1969). The work of the group of van der Waaij has already established that the anaerobic microflora together with host factors collaborate in providing resistance to colonization and/or infection (van der Waaij et al., 1971, 1977). Precisely how anaerobic bacteria go about controlling the colonization pattern of aerobes remains an open question: competition for nutrients (Donaldson, 1964), production of volatile fatty acids (Barnes et al., 1979) and bacteriocines (Booth et al., 1977) as well as stimulation of certain CR-related host activities such as peristalsis (Abrams and Bishop, 1967), production of mucin (Sprinz, 1962) and epithelial desquamation (Lesher et al., 1964), all are involved. The mechanisms by which the anaerobic part of the intestinal flora exerts

its inhibiting influence on newly arriving aerobic microorganisms is, in all likelihood, much more complex. This study has focused on this aspect of the intestinal flora in IBD patients. The CR has been measured in two ways. Firstly, by looking on the overall effect on aerobes: normal numbers of *Enterobacteriaceae* and enterococci were constantly found. Similarly the numbers of *St. aureus* and of *C. albicans* in the faeces were similar to that in normal adults as well as the carriage rate. Secondly, the β-aspartylglycine concentration in the faeces, i.e., the substance detecting disturbed anaerobic microflora was zero in all patients which also make likely that CR was intact in IBD patients. Some investigations report bacterial overgrowth (small bowel) in a small number of IBD patients, especially in Crohn patients. However, those cases were usually accompanied by complications like stasis, stenosed bowel or fistulae characteristic for Crohn's disease (Draser and Shiner, 1969; Prizont et al., 1970; Beeken and Kanich, 1973). These observations confirm that motility is undoubtedly important in maintaining normal aerobic bacterial population and plays a decisive role on the CR. Our 25 patients studied had comparatively normal gut motility. Limited diseased bowel segments (terminal ileum and/or colon) do not influence the resistance to colonization.

A normal CR implies a powerful barrier against single potentially pathogenic microorganisms trying to colonize the digestive tract. Given the fact that the CR function in IBD patients remains unimpaired when the disease is in relapse, it seems improbable that an exogenous environmental aerobic microorganism is involved. This could support Seneca and Henderson who in 1950 suggested that endogenous (resident) flora such as an autochtonous *Escherichia coli* strain, rather than any single 'as yet not discovered' microorganism, may play a key role in IBD.

ACKNOWLEDGEMENTS

We thank the nursing staff of the wards concerned for their collaboration. We want to express our appreciation to Mrs. G. Helmus and Mrs. E. van Santen for technical assistance and to Mrs. M.H. Brummer-Scholten for secretarial assistance.

REFERENCES

[1] SENECA, H., HENDERSON, E. (1950). Normal intestinal bacteria in ulcerative colitis. *Gastroenterology,* 15:34-39.

[2] WEINSTEIN, L. (1961). Bacteriological aspects of ulcerative colitis. *Gastroenterology,* 4:323-330.

[3] COOKE, E.M. (1967). A quantitative comparison of the faecal flora of patients with ulcerative colitis and that of normal persons. *J. Path. Bacteriol.,* 94:439-444.

[4] GORBACH, S.L., NAHAS, L., PLAUT, A.G., WEINSTEIN, L., PATTERSON, J.F., LEVITAN, R. (1968). Studies on the intestinal microflora: V. Fecal microbial ecology in ulcerative colitis and regional enteritis: relationship to severity of disease and chemotherapy. *Gastroenterology,* 54:575-587.

[5] VINCE, A., DYER, N.H., O'GRADY, F.W., DAWSON, A.M. (1972). Bacteriological studies in Crohn's disease. *J.Med.Microbiol.,* 5:219-229.

[6] VAN DER WIEL-KORSTANJE, J.A.A., WINKLER, K.C. (1975). The faecal flora in ulcerative colitis. *J.Med.Microbiol.,* 8:491-501.

[7] KEIGHLEY, M.R.B., ARABI, Y., DIMOCK, F., BURDON, D.W., ALLAN, R.N., ALEXANDER-WILLIAMS, J. (1978). Influence of inflammatory bowel disease on intestinal microflora. *Gut,* 19:1099-1104.

[8] VAN DER WAAIJ, D. (1971). Colonization resistance of the digestive tract in conventional and antibiotic mice. *J.Hyg.,* 69:405-411.

[9] VAN DER WAAIJ, D. (1979). The colonization resistance of the digestive tract in man and animals. *Zbl.Bakt.Suppl.,* 7:155-161.

[10] WELLING, G.W., GROEN, G., TUINTE, J.H.M., KOOPMAN, J.P., KENNIS, H.M. (1980). Biochemical effects on germ-free mice of association with several strains of anaerobic bacteria. *J.Gen.Microbiol.,* 117:57-63.

[11] VAN DER WAAIJ, D., BERGHUIS-DE VRIES, J.M. (1974). Determination of the colonization resistance of the digestive tract of individual mice. *J.Hyg.,* 72:379-387.

[12] WELLING, G.W., GROEN, G. (1978). β-aspartylglycine, a substance unique to caecal contents of germ-free and antibiotic-treated mice. *Bioch.J.,* 175:807-812.

[13] GORDON, H.A. (1960). The germ-free animal. Its use in the study of 'physiologic' effects of the normal microbial flora in the animal host. *Am.J.Dig.Dis.,* 5:841-867.

[14] ABRAMS, G.D., BAUER, H., SPRINZ, H. (1963). Influence of normal flora on mucosal morphology and cellular renewal in ileum: comparison of germ-free and conventional mice. *Lab.Invest.,* 12:355-364.

[15] DONALDSON, R.M. (1964). Normal bacterial populations of the intestine and their relation to intestinal function. *New Engl.J.Med.,* 270:938-945;994-1001.

[16] ABRAMS, G.D., BISHOP, J.E. (1967). Effect of the normal microbial flora on gastrointestinal motility. *Proc.Soc.Exp.Biol.Med.,* 126:301-304.

[17] FINGER, D., WOOD, W.B. Jr. (1955). The apparent activation of *Salmonella* enteritis by oxytetracycline. *Am.J.Med.,* 18:839-841.

[18] DUBOS, R., SCHAEDLER, R.W. (1962). Some biological effects of the digestive tract flora. *Am.J.Med. Sci.*,224:265-271.

[19] BONHOFF, M., MILLER, C.P., MARTIN, W.R. (1964). Resistance of the mouse's intestinal tract to experimental *Salmonella* infection. *J.Exp.Med.*, 120:805-816.

[20] ROSENTHAL, S.L. (1969). Exacerbation of *Salmonella* enteritis due to ampicillin. *New Engl.J.Med.*,280:147-148.

[21] BARNES, E.M., IMPEY, C.S., STEVENS, B.J.H. (1979). Factors affecting the incidence and anti-*Salmonella* activity of the anaerobic caecal flora of the young chick. *J.Hyg.*,82:263-283.

[22] BOOTH, S.J., JOHNSON, J.L., WILKINS, T.D. (1977). Bacteriocin production by strains of *Bacteroides* isolated from human feces and the role of these strains in the bacterial ecology of the colon. *Antimicrob.Agents Chemother.*,11:718-724.

[23] SPRINZ, H. (1962). Morphological responses of intestinal mucosa to enteric bacteria and its implication for Asiatic cholera. *Fed.Proc.*,21:57-64.

[24] LESHER, S., WALBURG, H.E., SACHER, G.A. (1964). Generation cycle in the duodenal crypt cells of germ-free and conventional rats. *Nature*,202:884-886.

[25] DRASAR, B.S., SHINER, M. (1969). Studies on the intestinal flora. Bacteriological flora of the small intestine in patients with gastrointestinal disorders. *Gut*,10:812-819.

[26] PRIZONT, R., HERSH, T., FLOCH, M.H. (1970). Jejunal bacterial flora in chronic small bowel disease. I. Celiac Disease II. Regional enteritis. *Am.J.Clin. Nutr.*,23:1602-1607.

[27] BEEKEN, W.L., KANICH, R.E. (1973). Microbial flora of the upper small bowel in Crohn's disease. *Gastroenterology*,65:390-397.

III

COLONIZATION RESISTANCE OF THE DIGESTIVE TRACT IN INFLAMMATORY BOWEL DISEASE PATIENTS

H.K.F. van Saene[1], W. Jansen[2], W.C. Veeger[3] and D. van der Waaij[4].

Laboratory for Medical Microbiology[1,4]
and Department of Gastroenterology[2,3],
University Hospital, Groningen, The Netherlands.

The colonization resistance of the digestive tract — as measure for the defense against bacterial colonization — was evaluated in 66 inflammatory bowel disease patients (36 with Crohn's disease and 30 with ulcerative colitis). This longitudinal qualitative and quantitative study of the aerobic flora indirectly revealed the intactness of the colonization resistance. The dipeptide β-aspartylglycine — only present when the anaerobic flora is affected — was not detected indicating that also the anaerobic part of the microflora shows no major differences. The fact that the colonization resistance functions normally in inflammatory bowel disease patients and the possible implications concerning the aetiology and pathogenesis of these diseases were discussed.

INTRODUCTION

Colonization resistance (CR) is defined as the resistance of an individual (or of the individuals digestive tract) against colonization by aerobic potentially pathogenic microorganisms (PPMs)[1]. CR is a

defense concept and a characteristic of an individual. CR of the digestive tract (DT) aims to prevent colonization of aerobic PPMs daily supplied by food and beverages. An aerobic PPM entering the DT by the oropharyngeal cavity tries to colonize the cavity, and after swallowing will try to adhere, to grow out and to colonize the intestines. The CR of the DT will inhibit these colonization attempts in order to prevent high concentrations of aerobic PPMs like *Enterobacteriaceae, St. aureus,* enterococci and yeasts. High concentrations of aerobic bacteria in the DT implies higher infection risks[2] and greater danger of translocation, i.e., passage of endogenous bacteria from the digestive tract lumen through the epithelial mucosa into the systematic immune systems (e.g., spleen). The concentration of aerobic *Enterobacteriaceae* determines whether or not e.g. *E. coli* will translocate[3].

Essential factors in the colonization resistance of the oropharynx are the integrity of the oral mucosa, mechanical cleansing by muscular actions of the tongue, cheeks and lips and by swallowing. This is greatly aided by saliva flow which in addition to lubricating the movements during chewing and swallowing makes it possible to swallow PPMs into the stomach[4]. Desquamation of epithelial cells, the antimicrobial components in saliva (lysozyme, peroxidase, lactoferrin, complement etc.)[5,6,7] as well as the indigenous oral flora (anaerobes, viridans streptococci)[8,9] function as a powerful defense system contributing to the oropharyngeal CR. The oral mucosa continuously bathing in saliva is covered by secretory immunoglobulin A (s-IgA) secreted with saliva. The current view of the mechanism of action of s-IgA is that the antibodies may combine with bacteria by 'coating' the microorganisms and prevent their adherence and subsequent colonization[10].

On the analogy of the oropharynx mechanical cleansing at intestinal level is ensued by peristalsis and mucin has the same lubricating function as saliva in the oral cavity[11,12]. Desquamation of mucosal cells to which bacteria adhere, s-IgA interfering with bacterial adherence and the anaerobic part of the microflora by its direct and indirect action against aerobes, all are involved in the control and/or elimination of aerobic PPMs[13,14].

This physiological CR factor can be measured in two ways: (i) by looking on the overall effect on aerobes. Normally, an oropharyngeal cavity is not colonized by aerobic *Enterobacteriaceae.* Only a transient stay in low concentrations is allowed. At intestinal level, low concentrations of *Enterobacteriaceae* ($< 10^6$ PPMs per gram of faeces) and short colonization periods of newly acquired aerobes imply an intact CR. (ii) The resistance to colonization — due to the anaerobic microflora — can be directly measured by a test detecting β-aspartylglycine, a dipeptide appearing in cases of disturbed anaerobic flora[15]. Endogenous proteins and proteins from the diet are continously degraded to amino acids by proteolytic enzymes from the host and the bacteria in the intestinal tract. When the anaerobic microflora is affected β-aspartylglycine accumulates in the faeces. Its unusual peptide bond can only be cleaved by bacterial enzymes which will result in aspartic acid and glycine. β-aspartylglycine is absent in stools of individuals with a normal bowel flora.

It is in the light of these considerations that we focus in this study on the oropharyngeal and faecal flora of 66 patients with inflammatory bowel disease (IBD) (36 with Crohn's disease and 30 with ulcerative colitis).

PATIENTS AND METHODS

Patients

Patients included in this study were all in-patients of the gastroenterology department of the University Hospital. The 66 patients were all adults with a clinical diagnosis of inflammatory bowel disease (36 with Crohn's disease and 30 with ulcerative colitis). They entered the study only if they were likely to have a prolonged hospital stay and if they had not had antimicrobial agents for at least one month before hospitalization. The necessity to use antimicrobial agents during the study led to the exclusion from this study.

Colonization resistance

Oropharyngeal swabs and faecal specimens were obtained twice weekly for six consecutive weeks. Each specimen was processed within 30 minutes of sampling.

1. oropharynx

monitoring of aerobic flora

The posterior pharynx of each subject was streaked with a cotton-tipped swab. Three agar plates were then corner streaked immediately with the swab, and its tip was broken off into broth. Cultures were done on sheep blood agar (Oxoid), MacConkey agar (Oxoid), yeast isolation agar (Merck) and in brain heart infusion (BHI) broth (Oxoid). All agar plates were incubated at 37° C; Mac Conkey agar plates were examined after one night, yeast isolation and sheep blood agar plates after two nights. The aerobic bacteria in all cultures were qualitatively and semi-quantitatively estimated. The normal indigenous oropharyngeal flora (viridans streptococci) were identified by colonial morphology and type of haemolysis on blood agar. The other bacteria (*St. aureus, Enterobacteriaceae, Pseudomonadaceae* and yeasts) were identified by means of standard bacteriological techniques[16]. Semi-quantitative estimation of all these bacteria was made on a scale of +1 to +5 according to their presence in broth (+1) and growth density in the four quadrants of the agar plates (+2 to +5).

2. intestinal tract

— monitoring of aerobic flora

Concentrations of aerobic gram-negative bacilli, yeasts and enterococci were determined as follows: one gram of faeces was homogenized in 9 ml of BHI, serially diluted (1:10) and incubated for 18 hours at 37° C; thereafter, all dilutions showing bacterial growth were inoculated onto MacConkey agar, yeast isolation agar and kanamycin aesculin azide agar (Oxoid). Concentrations of staphylococci in faecal samples were measured by a serial dilution in a salt containing liquid medium (10% NaCl, Oxoid). After incubation all dilutions were subcultured on sheep blood agar. Identification and typing was performed by means of standard bacteriological techniques. All results were expressed as the $\log_{10}$ of the number of

organisms per gram (wet weight) of faeces.

— β-aspartylglycine

The techniques for β-aspartylglycine detection in faeces are previously described. In brief, a 25 % w/v faecal suspension was centrifuged for 15 minutes at 15,000 rpm. 80 µl of the supernatant was subjected to high voltage paper electroforesis at pH 3.5. After staining with ninhydrin and drying at 150° C the paper was examined for the presence of a clear blue spot of β-aspartylglycine.

Definitions

— resident and transient *E. coli* strains:

'The *E. coli* flora of the human bowel is made up of two kinds of strains, those which establish themselves firmly and continue to multiply over extended periods of time and those which are found only in a single or a few successive specimens. It is convenient to speak of these two kinds of strains as residents and transients respectively.'[17].

— carrier:

in this study we defined a carrier as a patient where an identical PPM was cultured in more than 60 % of the samples during the six week study.

RESULTS

Normal colonization resistance (for comparison)

Figure 1 shows a colonization pattern of the digestive tract of *Enterobacteriaceae* in a normal subject. All newly acquired *Enterobacteriaceae* are eliminated from the oropharynx and the gut; they are able to colonize the digestive tract in low concentrations for short periods.

β-aspartylglycine is absent in stools of individuals with a normal bowel flora.

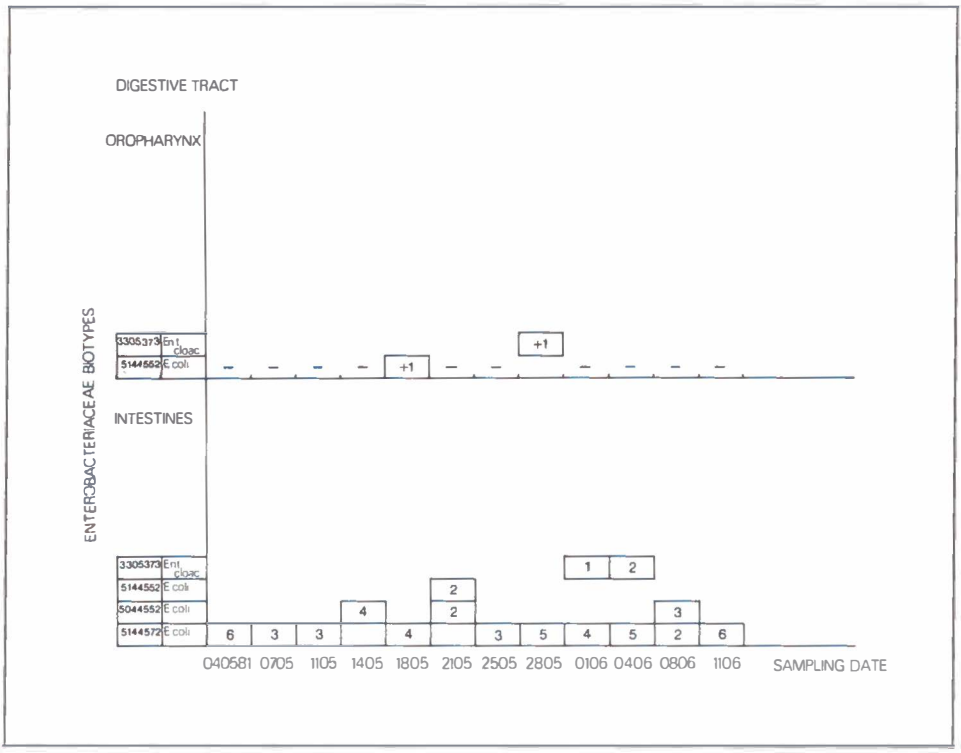


FIGURE 1

Normal colonization pattern of *Enterobacteriaceae* of digestive tract. Numbers in blocks give the semi-quantitative estimation of oropharyngeal concentrations of *Enterobacteriaceae* and the concentration of *Enterobacteriaceae* expressed as the $\log_{10}$ of bacteria per gram of faeces.

Composition of the aerobic flora in IBD patients

Longitudinal (minimum 6 weeks) qualitative and quantitative culturing of 767 digestive tract samples (767 oropharyngeal as well as faecal samples) (11.6 samples per individual) was carried out. Figure 2 shows *Enterobacteriaceae* colonization pattern of the digestive tract of a patient with Crohn's disease (a) and of a patient with ulcerative colitis (b).

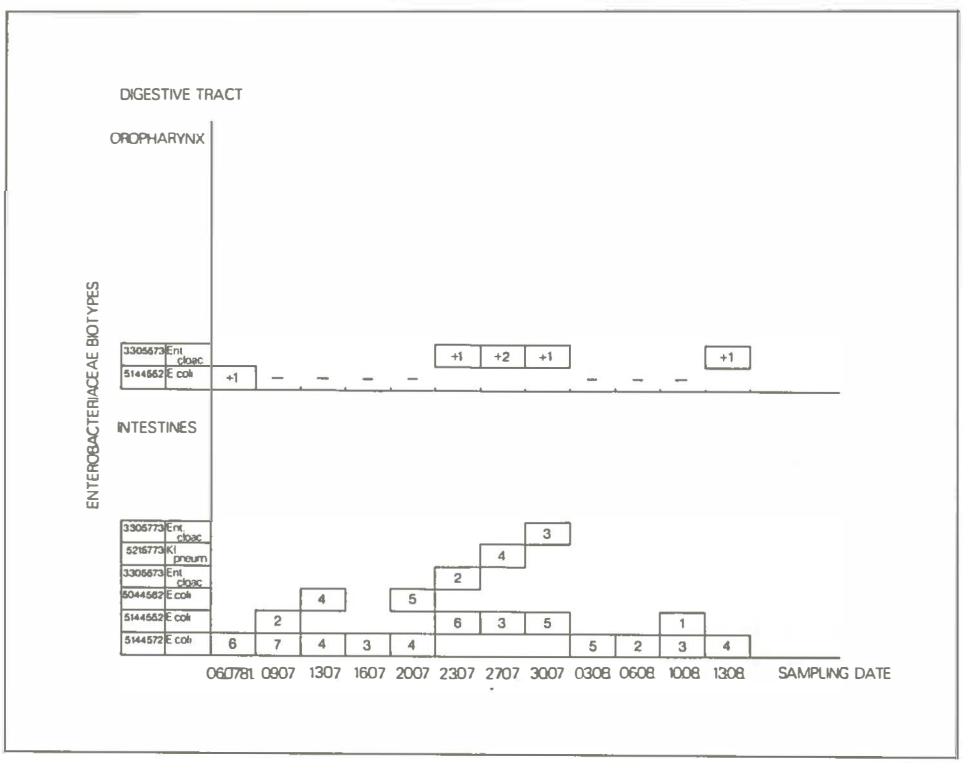


FIGURE 2

a. Colonization pattern of *Enterobacteriaceae* of digestive tract in a Crohn's disease patient.

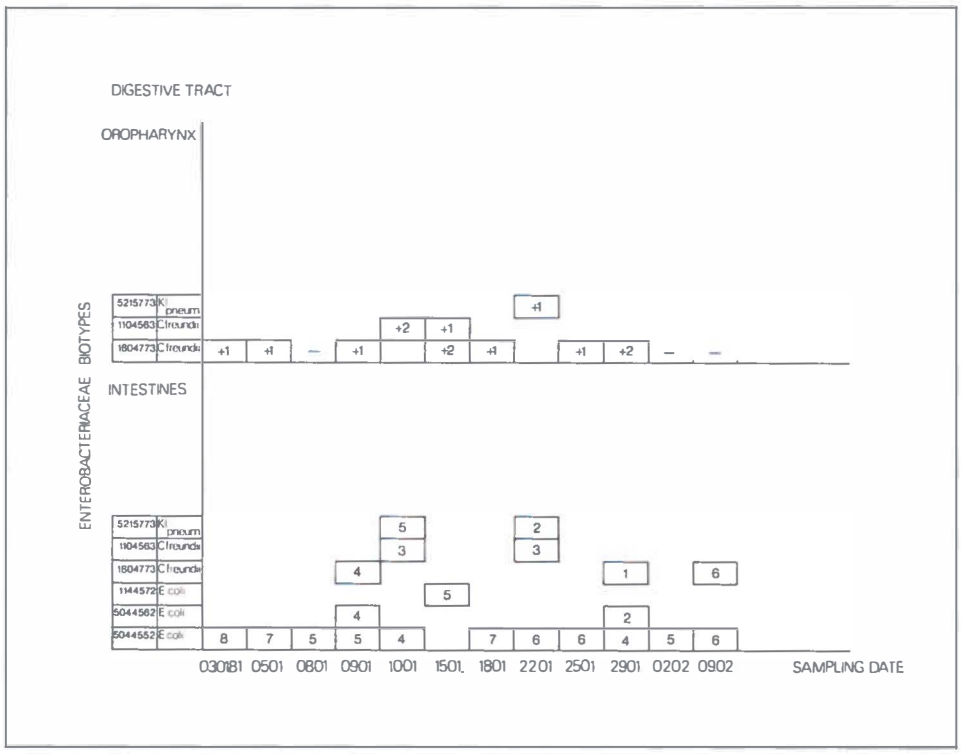


FIGURE 2
b. Colonization pattern of *Enterobacteriaceae* of digestive tract in an ulcerative colitis patient, who was oropharyngeal carrier.

1. Oropharynx

The prevalence of aerobic PPMs in the oropharyngeal cultures of IBD patients is shown in Table 1. The percentages found were within the normal limits. The Gram-negative bacilli isolated from the oropharynx all belonged to the *Enterobacteriaceae* spp. *Pseudomonadaceae* could be never detected. With the exception of viridans streptococci, the total number of colonies of all species observed on the agar plates was small. The mean growth densities varied from +1.3 to +1.8, i.e., only in broth medium and in the first quadrant PPMs were grown.

TABLE 1
Percentage of oropharyngeal and faecal cultures containing potentially pathogenic microorganisms.

Potentially pathogenic microorganisms	Digestive tract	
	Oropharynx	Intestinal tract
St. aureus	27	32
Enterobacteriaceae	14	100
Pseudomonadaceae	—	—
C. albicans	18	17
oral streptococci	100	—
faecal streptococci	—	100

2. Intestines

The intestinal flora in IBD patients was similar to that in normal individuals. The resident flora included *E. coli* only (two bioprofiles). The transient flora consisted of all representatives of the *Enterobacteriaceae* family, with the exception of *Salmonella* spp. *Pseudomonas* spp were again not detected. The concentrations of aerobic PPMs remained approximately constant during the study period (10^4 - 10^7 bacteria per gram of faeces). Out of the 66 inflammatory bowel disease patients twenty patients (33 %) (10 with Crohn's disease and 10 with ulcerative colitis) showed a faecal concentration of $\geqslant 10^6$ *Enterobacteriaceae* per gram of faeces. This percentage of 33 % was the same as the percentage found in normal individuals.

β-aspartylglycine as measure of the anaerobic faecal flora

No β-aspartylglycine could be found in the faecal specimens of the 66 patients.

Carriership

The carriage rate of *St. aureus* (12/66 in the oropharynx and 16/66 in the intestines) was relatively high but within normal limits. The rate for *Candida albicans* and *Enterobacteriaceae* (*E. coli, Klebsiella* spp, *Citrobacter* spp and *Enterobacter* spp) was within normal range (Table 2). An oropharyngeal carrier of *Citrobacter freundii* is represented in Figure 2-b-.

TABLE 2

Percentage of patients with inflammatory bowel disease who were carriers of potentially pathogenic microorganisms. Figures between brackets indicate normal values of carriership.

Potentially pathogenic microorganisms	Digestive tract			
	Oropharynx		Intestinal tract	
St. aureus	18	(10-40)	24	(10-40)
Enterobacteriaceae	8	(⩽10)	100	(⩽100)
Pseudomonadaceae	—	(⩽1)	—	(⩽1)
C. albicans	15	(10-40)	14	(10-40)
oral streptococci	100	(⩽100)	—	
faecal streptococci	—		100	(⩽100)

Colonization resistance

Colonization pattern, absence of β-aspartylglycine and carriage rate show that in both groups (Crohn's disease and ulcerative colitis) the colonization resistance as measured remained intact.

DISCUSSION

The essential factor contributing to the colonization resistance of the digestive tract is the presence of the anaerobes. Precisely how anaerobic bacteria go about controlling the colonization pattern of aerobes remains an open question: competition for nutrients[18], production of volatile fatty acids[19] and bacteriocines[20] as well as stimulation of certain CR-related host activities such as peristalsis, production of mucin and epithelial desquamation, all are involved. The mechanisms by which the anaerobic part of the digestive tract flora exerts inhibiting influence on newly arriving aerobic microorganisms is , in all likelihood, much more complex. This study has focused on the CR supporting activity of the anaerobic part of the microflora in the digestive tract. It is very likely that not all anaerobic species are as important concerning their contribution to the CR of the DT[21]. Some investigators found that the prevalence of some anaerobic species (*Peptostreptococcus* and *Eubacterium*) was higher in Crohn's disease patients as compared with controls[22]. In the same study the aerobic colonization pattern was completely normal. We found that the CR of the DT in IBD patients functions normally. The aerobic digestive tract flora qualitatively and quantitatively does not differ from normal flora. Also the CR supporting activity of the anaerobic flora remains intact, indicating that also the anaerobic flora shows no major differences. The carriage rates of *Enterobacteriaceae, St. aureus* and *C. albicans* were similar to that found in normal subjects[23,24,25]. Figure 2.b shows the colonization pattern of an ulcerative colitis patient who was also an oropharyngeal carrier of *Enterobacteriaceae.* This patient was severely ill and her oropharyngeal mucosa may have been modified probably due to her severly catabolic state resulting in an enhanced adherence by Gram-negative bacilli[26].

Because of the intactness of the CR resulting in normal concentrations of aerobic PPMs, IBD patients rarely develop infections. Post-operative wound healing problems can arise sporadically: *St. aureus* is then practically always the microorganism involved. Also the adherence of *St. aureus* is probably increased in this type of patient as a consequence of the modified mucosa. A second consequence of an

intact CR concerns the aetiopathogenesis of IBDs. There is considerable evidence for the involvement of an auto-immunological process[27]. A possibility is an altered immune reactivity to digestive tract-associated antigens, including enterobacterial antigens[28]. In other words, the patient could be hypersensitized (circulating antibodies) to his endogenous *Enterobacteriaceae* following bacterial translocation. Two mechanisms inhibit translocation of indigenous *Escherichia coli:* bacterial antagonism[29] and local immunity[30]. Bacterial antagonism is an important component of the colonization resistance. For, the anaerobic part of the microflora controls the colonization pattern of indigenous *E. coli* and possible subsequent translocation[31]. The contribution of local immunity to translocation of endogenous *E. coli* is studied in athymic mice which do not produce secretory immunoglobulin A. Restoration of local immunity inhibited translocation. If translocation plays a role in the pathogenesis of IBDs by inducing of circulating anti *E. coli* antibodies, a dysfunction of local immunity seems more likely than a disturbance of CR to explain elevated anti *E. coli* titres.

A normal CR implies a powerful barrier against single potentially pathogenic microorganisms trying to colonize the digestive tract. Given the fact that the CR function in IBD patients remains unimpaired when the disease is in relapse, it seems also improbable that an exogenous environmental aerobic microorganism is involved. This supports Seneca and Henderson who in 1950 suggested that endogenous (resident) flora such as an autochtonous *Escherichia coli* strain, rather than any single 'as yet not discovered' microorganism may play a key role in IBD[32]. It is of interest also to recall the statement of Kirsner and Palmer that not a specific potentially pathogenic microorganism (Mycobacterium, virus etc.) may be involved, but rather the concept of the vulnerability of the host must be focused upon[33]. Evidence in the literature suggests that this vulnerability may include a dysfunction of local immunity resulting in a translocation of the endogenous *E. coli* leading to circulating antibodies with which the hypersensitized IBD patients breaks down his own digestive tract[34,35,36,37,38,39].

ACKNOWLEDGEMENTS

We are indebted to the nursing staff for their cooperation in obtaining specimens. We gratefully acknowledge the skilful technical assistance of Mrs. G. Helmus, Mrs. E. van Santen and Mr. J. Krikken and the excellent secretarial assistance of Mrs. S. Swart.

REFERENCES

[1] VAN DER WAAIJ, D., BERGHUIS-DE VRIES, J.M. (1974). Determination of the colonization resistance of the digestive tract of individual mice. *J.Hyg.*, 72:379-387.

[2] SCHIMPFF, S.C. (1980). Infection prevention during profound granulocytopenia. *Ann.Int.Med.*,23:358-361.

[3] BERG, R.D., GARLINGTON, A.W. (1979). Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymphnodes and other organs in a gnotobiotic mouse model. *Infect.Immun.*,23:403-411.

[4] LAFORCE, F.M., HOPKINS, J., TROW, R., WANG, W.L.L. (1976). Human oral defenses against Gram-negative rods. *Am.Rev.Respir.Dis.*,114:929-935.

[5] BJÖRNESÖ, K.B. (1950). Studies on the antibacterial factors of human saliva. *Acta Chem.Scand.*,4:835-845.

[6] KLEBANOFF, S.J., CLEM, W.H., LUEBKE, R.G. (1966). The peroxidase-thiocyanate-hydrogen peroxide antimicrobial system. *Biochem.Biophys.Acta,* 117:63-72.

[7] ROITT, I.M., LEHNER, T. (1980), Oral Immunity. In: *Immunology of oral diseases.* Eds. Roitt, I.M., Lehner, T. Blackwell, Oxford,297-322.

[8] MURRAY, P.R., ROSENBLATT, J.E. (1976). Bacterial interference by oropharyngeal and clinical isolates of anaerobic bacteria. *J.Inf.Dis.*,134:281-285.

[9] SPRUNT, K., LEIDY, G.A., REDMAN, W. (1971). Prevention of bacterial overgrowth. *J.Inf.Dis.*,123:1-10.

[10] WILLIAMS, R.C., GIBBONS, R.J. (1972). Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. *Science,* 177:697-698.

[11] ABRAMS, G.D., BISHOP, J.E. (1967). Effect of the normal microbial flora on gastrointestinal motility. *Proc.Soc.Exp.Biol.Med.*,126:301-304.

[12] SPRINZ, H. (1962). Morphological responses of intestinal mucosa to enteric bacteria and its implication for Asiatic cholera. *Fed.Proc.*,21:57-64.

[13] LESHER, S., WALBURG, H.E., SACHER, G.A. (1964). Generation cycle in the duodenal crypt cells of germ-free and conventional rats. *Nature,*202:884-886.

[14] FRETER, R. (1974). Interactions between mechanisms controlling the intestinal microflora. *Am.J.Clin.Nutr.*,27:1409-1416.

[15] WELLING, G.W., GROEN, G. (1978). β-aspartylglycine, a substance unique to caecal contents of germ-free and antibiotic treated mice. *Bioch.J.*,175:807-812.

[16] SMITH, P.B., TOMFOHRD, K.M., RHODEN, D.L., BALOWS, A. (1972). API system: a multitube micromethod for identification of *Enterobacteriaceae*. *Appl.Microbiol.*,24:449-452.

[17] ROBINET, H.G. (1962). Relationship of host antibody to fluctuations of *Escherichia coli* serotypes in the human intestine. *J.Bacteriol.*,84:896-901.

[18] DONALDSON, R.M. (1964). Normal bacterial populations of the intestine and their relation to intestinal function. *New Engl.J.Med.*,270:938-945.

[19] LEE, A., GEMMELL, E. (1972). Changes in the mouse intestinal microflora during weaning: role of volatile fatty acids. *Infect.Immun.*,5:1-7.

[20] BOOTH, S.J. JOHNSON, J.L., WILKINS, T.D. (1977). Bacteriocin production by strains of *Bacteroides* isolated from human feces and the role of these strains in the bacterial ecology of the colon. *Antimicrob.Agents Chemother.*,11:718-724.

[21] ALUWIHARE, A.P.R. (1971). An ultrastructural study of the effect of neomycin on the colon in the human subject and in the conventional and the germ-free mouse. *Gut*,12:341-349.

[22] WENSINCK, F. (1976). Faecal flora of Crohn's patients. Serological differentiation between Crohn's disease and ulcerative colitis. In: *The management of Crohn's disease*. Eds. Weterman, I.T., Pena, A.S. Booth, C.C. Excerpta Medica, Amsterdam,103-105.

[23] ROSENTHAL, S., TAGER, I.B. (1975). Prevalence of Gram-negative rods in the normal pharyngeal flora. *Ann.Int.Med.*,83:355-357.

[24] MILLIAN, S.J., BALDWIN, J.N., RHEINS, M.S., WEISER, H.N. (1960). Studies on the incidence of coagulase positive staphylococci in a normal unconfined population. *Am.J.Public Health*,50:791-798.

[25] BARLOW, A.J.E., CHATTAWAY, F.W. (1969). Observations on the carriage of *Candida albicans* in man. *Br.J.Dermatol.*,81:103-106.

[26] JOHANSON, W.G., PIERCE, A.K., SANFORD, J.P. (1969). Changing pharyngeal bacterial flora of hospitalized patients. *New Engl. J.Med.*, 281:1137-1140.

[27] JEWELL, D.P., HODGSON, H.J.F. (1976). Auto-immune and inflammatory diseases of the gastrointestinal tract. In: *Immunological aspects of the liver and gastrointestinal tract*. Eds. Ferguson, A., Mac Sween, R.N.M. Medical and Technical Press, Lancaster, 203-250.

[28] SHORTER, R.G. (1974). Immunological aspects of gastrointestinal disease: an up-to-date account of inflammatory diseases such as ulcerative colitis and Crohn's disease. In: *Progress in Immunology* II. Eds. Brent, L., Holborow, J. North-Holland Publishing Company, Amsterdam,4:209-219.

[29] BERG, R.D., OWENS, W.E. (1979). Inhibition of translocation of viable *Escherichia coli* from the gastrointestinal tract of mice by bacterial antagonism. *Infect.Immun.*,25:820-827.

[30] OWENS, W.E., BERG, R.D. (1980). Bacterial translocation from the gastrointestinal tract of athymic (nu/nu) mice. *Infect.Immun.*, 27:461-467.

[31] VAN DER WAAIJ, D., TIELEMAN-SPELTIE, T.M., DE ROECK-HOUBEN, A.M.J. (1978). Relation between the faecal concentration of various potentially pathogenic microorganisms and infections in individuals (mice) with severely decreased resistance to infection. *Antonie van Leeuwenhoek,*44:395-405.

[32] SENECA, H., HENDERSON, E. (1950). Normal intestinal bacteria in ulcerative colitis. *Gastroenterology,*15:34-39.

[33] KIRSNER, J.B., PALMER, W.L. (1954). Ulcerative colitis - considerations of its etiology and treatment. *J.Am.Med.Assoc.,*155:341-346.

[34] GELZAYD, E.A., KRAFT, S.C., FITCH, F.W., KIRSNER, J.B. (1968). Distribution of immunoglobulins in human rectal mucosa. II. Ulcerative colitis and abnormal mucosal control subjects. *Gastroenterology,*54:341-347.

[35] DAS, K.M., ERBER, W.F., RUBINSTEIN, A. (1977). Immunohistochemical changes in morphologically involved and uninvolved colonic mucosa of patients with idiopathic proctitis. *J.Clin.Invest.,*59:379-385.

[36] ENGSTROM, J.F., ARVANITAKIS, C., SAGAWA, A., ABDOU, N.I. (1978). Secretory immunoglobulin deficiency in a family with inflammatory bowel disease. *Gastroenterology,*74:747-751.

[37] STROBER, W., KRAKAUER, R., KLAEVEMAN, H.L., REYNOLDS, H.Y., NELSON, D.L. (1976). Secretory component deficiency. A disorder of the IgA immune system. *New Engl.J.Med.,*294:351-356.

[38] CHILGREN, R.A., MEUWISSEN, H.J., QUIE, P.G., HONG, R. (1967). Chronic mucocutaneous candidiasis, deficiency of delayed hypersensitivity and selective local antibody defect. *Lancet,*ii:688-693.

[39] GREEN, F.H.Y., FOX, H. (1975). The distribution of mucosal antibodies in the bowel of patients with Crohn's disease. *Gut,*16:125-131.

CHAPTER FOUR

LOCAL IMMUNE SYSTEM

„secretory immunoglobulin A
is the antiseptic paint for mucosal surfaces"

Burnet, F.M.

I

A NOVEL TECHNIQUE FOR DETECTING IgA COATED POTENTIALLY PATHOGENIC MICROORGANISMS IN THE HUMAN INTESTINE

H.K.F. van Saene and D. van der Waaij.

Laboratory for Medical Microbiology,
University Hospital, Groningen, The Netherlands.

This paper describes a novel method for detecting immunoglobulin A (IgA) coated potentially pathogenic microorganisms (PPMs) in the human intestine. Essentially, the technique consists of 2 phases: one in which IgA coated bacteria are detected by immunofluorescence and a second in which these bacteria are subcultured in situ and subsequently identified. In this way transient bacteria are differentiated from resident bacteria. These results show that the resident bacteria are coated with IgA. Resident microorganisms are always highly concentrated in the digestive tract. These results strengthen the hypothesis that only the high antigen concentrations achieved by a large number of resident bacteria are capable of IgA induction.

INTRODUCTION

The functions of the immunoglobulin A (IgA) system of the intestine are discussed by Heremans (1975). One of these is presumably prevention of colonization of the digestive tract (DT) by certain potentially pathogenic microorganisms (PPMs). PPMs attempting to colonize the DT are assumed to attach or adhere to DT epithelium.

The anticolonization function of the IgA system of the DT possibly involves inhibition of this adherence or attachment by surrounding or coating the PPMs with IgA molecules (Gibbons, 1974). In experiments in mice, Van der Waaij and Heidt (1977) showed that only resident aerobic bacteria are capable of IgA induction. A resident bacterium is a PPM remaining in the tract for more than one week. A transient organism is a PPM which occurs only sporadically in the tract, e.g., once in 2 months. Resident bacteria tend to colonize; transient ones never do. Only aerobic PPMs *(Enterobacteriaeceae,* yeasts, etc.) possess immunogenic IgA inducing properties.

This report describes the relationship between IgA production and the colonization pattern of *Enterobacteriaceae* species in the human intestine. The colonization pattern reflects differentiation between transient and resident PPMs. As representatives of the group of potentially pathogenic microorganisms, only the *Enterobacteriaceae* species are included in this study. Other PPMs such as staphylococci, streptococci, yeasts and fungi are not considered because they often give false positive staining with immunofluorescent anti-IgA. Typing is necessary in order to differentiate resident from transient bacteria. This novel technique for detecting IgA coated bacteria is illustrated by the results obtained with a group of healthy volunteers.

MATERIALS AND METHODS

Preparation of homogeneous faecal suspension

One gram of faeces together with 9 ml of phosphate buffered saline (PBS) was suspended in a mortar. After centrifugation (450 x *g* for 30 min at 4° C), the supernatant representing a 1 : 10 diluted faecal suspension was retained.

Direct fluorescence staining in suspension

This 10^{-1} faecal suspension included IgA coated (IgA+) and non-IgA coated (IgA—) *Enterobacteriaceae.* With a fluorescent antiserum, goat antihuman (GAHU) IgA labelled with fluorescein isothiocyanate (FITC) (Nordic Immunological Laboratories, Tilburg,

The Netherlands), IgA + *Enterobacteriaceae* were detected. A 0.10 ml sample of the 10^{-1} faecal suspension was incubated together with 0.10 ml of conjugate for 45 min at 4° C. Optimal conjugate dilution was between 1 : 4 and 1 : 16. After incubation, the 0.2 ml fluorescent faecal suspension was mixed with 9.8 ml of PBS, resulting in a 1 : 1000 faecal suspension.

Preparation of the slides

(a) Fat-free slides (76 x 26 mm, Menzel Gläzer, F.R.G.) were covered with a thin layer of 5 ml liquid sterilized MacConkey agar (MC agar) (Merck, Art 5465, Darmstadt, F.R.G.). A stabilisation period of 1 h at room temperature on a horizontal surface yielded a smooth and dry surface.

(b) Two droplets of 0.05 ml each of the fluorescent 10^{-3} faecal suspension were placed on this medium. When the two droplets had soaked into the agar, the preparation was covered with a large coverslip (24 x 60 mm, Menzel Gläzer, F.R.G.) and placed in a glass petri dish with moistened filter paper. Until examined, the IF slides were kept in a refrigerator at 4° C.

Microscopic equipment

Immunofluorescence (IF) and phase contrast microscopy (Carl Zeiss Type IV F, F.R.G.) were employed. The filter system was standard, with an excitation (455—190 nm) and an emission (520—560 nm) filter, and CPLW 10x were chosen as eyepieces. The objectives were a Planapo PL 3 40x (with immersion oil) and a 10x Planapo (without immersion oil).

Microscopic detection and localization of IgA coated bacteria

The microscope was first focused by phase contrast: rod shaped bacteria situated on the surface of the agar were clearly detected at the level of the drop areas, the circular agar areas originating from the application of the fluorescent faecal sample. When the microscope was focused, the two drop areas (two faecal droplets) were screened for IgA + (fluorescence +) bacteria by IF. IgA + rods detected were registered on a special form and recorded by means of a coordinate

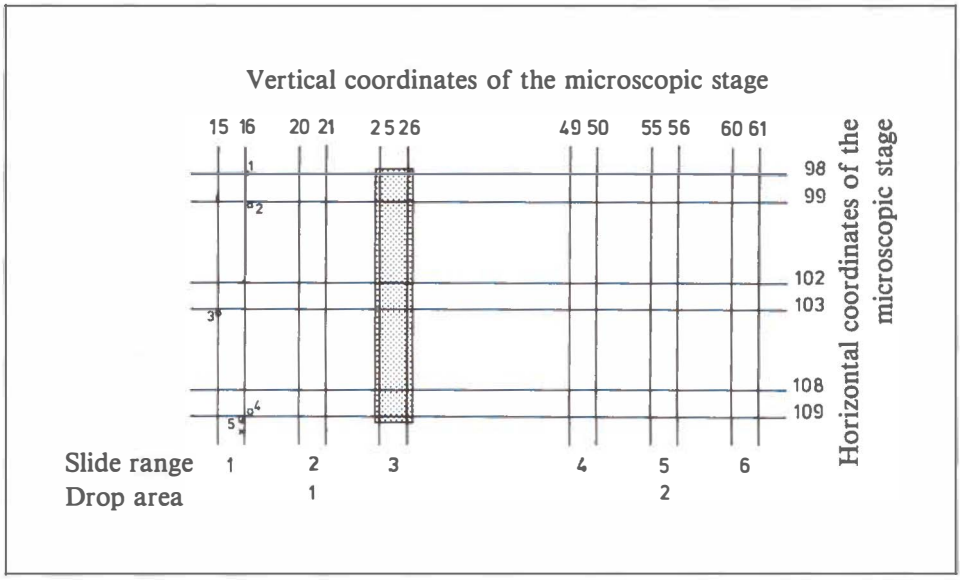


FIGURE 1
Sketch representing coordinate grating, consisting of 72 microscopic fields (points on intersection). A slide comprises 2 drop areas and 6 slide ranges. Slide range 3 is shaded. Black lines represent IgA coated rods; circled areas represent *Enterobacteriaceae* colonies.

grating consisting of 6 horizontal and 12 vertical lines. The millimeter scale of the microscope stage provided lines at right angles to each other, in this way constructing the precise network of the grating. A point of intersection determined a particular microscopic field, and 6 x 12 (i.e. 72) microscopic fields evenly distributed over the slide allowed uniform screening of the two faecal droplets.

Figure 1 is a sketch of the coordinate grating. To facilitate interpretation an adjustment procedure was found to be necessary before completing the form. On exceptional occasions when an IgA+ rod was detected in the middle of a microscopic field, it was registered at the appropriate point of intersection. Rods were normally non-centrally localized, and the microscopic field was then carefully adjusted to bring the fluorescent rod to the centre of the field. This modified position was registered on the form. In Figure 1 (drop area 1) in microscopic field 109, 16, an eccentric IgA+ rod is shown, lower left. Several IgA+ bacteria were observed in this way in each field; all were recorded. It generally required about 20 min to examine an IF slide containing a large number of coated bacteria.

In situ subculturing

After the IF examination, the slide (with cover slip) was placed in a petri dish with moistened filter paper and incubated at 37° C for 18 h.

Microscopic localization of the colonies

Colonies were seen under the cover slip after incubation and were small because of the restraining influence of the cover slip. The probability that these colonies were *Enterobacteriaceae* was very high, because of the selective medium (MC agar). Both IgA+ and IgA— bacteria formed colonies since coating neutralizes but does not kill a bacterium (Heremans, 1974).

After removal of the cover slip, the *in situ* subcultured faecal slide was again examined microscopically. This stage of the technique required only phase contrast (eyepieces, CPL 10x; objective, 1x without immersion oil). Compared with the IF stage, the lens combination now gave 4 times less magnification. To localize all

Enterobacteriaceae colonies, the coordinate grating of 72 fields was again used. The adjusting procedure described above was again used for phase contrast examination: before recording a colony, it was localized in the middle of the field. To illustrate this, Figure 1 (drop area 1, in field 109, 16) shows two colonies detected by this 10 x 10 combination (lower left and upper right of the point of intersection). These two colonies were recorded — after adjustment — on the form used for IgA + bacteria on the previous day.

Interpreting the microscopy results

The registration form thus consists of the findings at the two stages of the technique, namely, localization of IgA + bacteria, and of all colonies originating from IgA + as well as IgA— bacteria. These data permitted determination of which colonies were the progeny of IgA + bacteria. In the same example (Figure 1), the colony localized lower left to the point of intersection had originated from an IgA + bacterium detected by fluorescence at that level on the previous day. This conclusion was indicated by a special mark, e.g., a cross. The colony observed at the upper right was registered where no fluorescent rod had been seen. As the first microscopic examination involved screening by IF only, an IgA— bacterium must have been located at that site.

Identification of Enterobacteriaceae colonies

All colonies originating from IgA + and IgA— *Enterobacteriaceae* were identified and biotyped. The typing system used was the API system (La Balme, France). Before starting the API procedure, all colonies were coded and cultured to obtain purity.

a. Coding colonies

For practical reasons, the colonies were numbered. To achieve this, the concept of slide range was introduced. A slide range — a narrow ship — covers 12 microscopic fields vertically. Thus, a faecal sample consists of 6 vertical bandlike slide ranges, 3 per drop area. In Figure 1, slide range, the *Enterobacteriaceae* colonies were numbered from the top downwards. In slide range 1 of drop area 1, five colonies were recorded after 18 h of incubation. More colonies were present in

each slide range, but only those which were examined in relation to the 72 microscopic fields were considered. Interpretation led to the finding that 4 of the 5 colonies shown in Figure 1 have originated from IgA— bacteria, the other would have been the progeny of an IgA+ bacterium. Numbering from the top downwards, this colony became number 5 and is indicated in the figure by a cross.

b. Pure-culturing of coded colonies.

All numbered colonies were carefully picked from the MC agar and pure-cultured by standard bacteriological techniques.

c. Identification and typing of coded colonies.

. The procedure consisted of inoculating an API strip with a suspension prepared from a pure culture colony. After incubation, the reactions were recorded to give a bionumber. Finally, the API index transformed the bionumber into genus and species (Myerowitz, 1977).

Controls

a. Controls for the fluorescence part of the technique

Three possibilities of nonspecific fluorescence arise. In the conjugate, there may be IgG molecules not only against IgA but also against IgM and IgG, i.e., the conjugate is heterospecific. Secondly, the presence of antibodies against *Enterobacteriaceae* could lead to nonspecific fluorescence. Thirdly, *Enterobacteriaceae* possibly possess Fc receptors to which the specific AHU IgA, IgG molecules adhere by their Fc part ('sticky' effect) instead of binding with the Fab part of the molecule. Testing for monospecificity was carried out with myeloma cells, IgA, IgG and IgM. Three conjugate dilutions were tested: 1 : 5, 1 : 10 and 1 : 20. The other two possibilities were tested in the following way. *In situ* subculturing indicated that an IgA+ bacterium had formed a colony of 10^9 uncoated bacteria. After pure culturing and preparation of a suspension, two droplets of 0.05 ml of the suspension were incubated together with two droplets of 0.05 ml of conjugate.

b. Controls for *Enterobacteriaceae* survival during the procedure

Incubation with conjugate, and the microscopic examination, possibly impaired *Enterobacteriaceae* survival. For this reason, an untreated fraction of the same sample was used as control and was in-

cubated with PBS instead of conjugate, while the microscopic procedure was replaced by leaving the slide for 20 min at room temperature. The suspension was monitored by inoculating an MC agar plate with 0.05 ml. Control plates were also incubated for 18 h. The next day, the colonies on the control plates and on the slides with MC agar were counted and compared.

c. Controls for the faecal sample inventory

Only after the typing stage was control possible to determine how 'complete' the inventory of *Enterobacteriaceae* biotypes had been. The number of codified pure cultured and identified colonies had to be sufficient to ensure that no biotype was missing. For this purpose, an 'inventory curve' (Van der Waaij, 1974) was utilized.

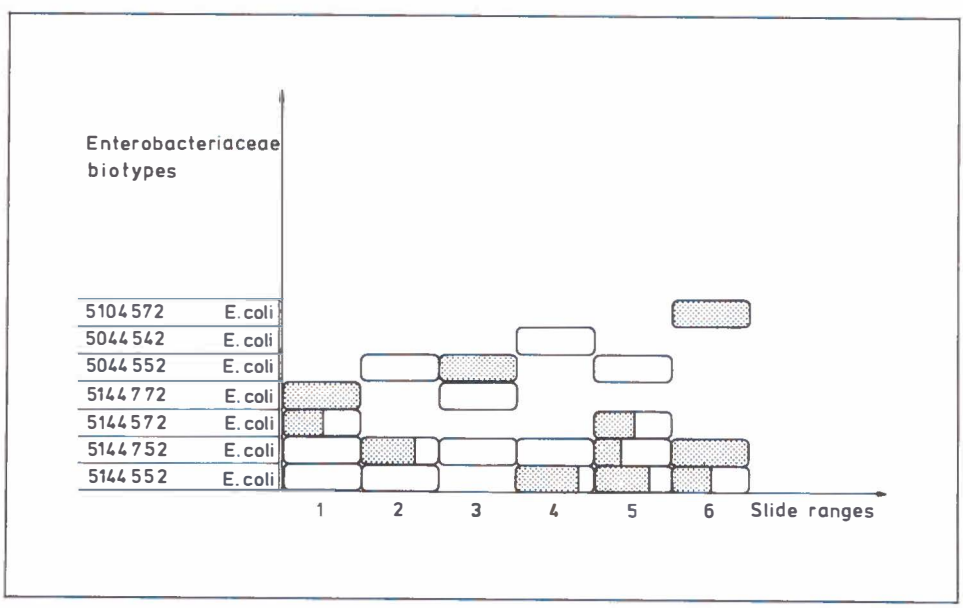


FIGURE 2

Inventory of faecal sample taken 24/3 (Figure 4). Number of different *Enterobacteriaceae* biotypes detected in the 6 slide ranges. The fraction of IgA + bacteria is proportionally represented by shading.

Samples

Four laboratory co-workers were sampled twice a week for approximately 2 months. Faecal samples were investigated within 1 h after defaecation. If immediate examination was not possible, the samples were frozen in liquid nitrogen.

RESULTS

Figures 2-4 show how results were recorded. Figures 2 and 3 are the inventory results of the same faecal sample of volunteer 1. Figure 4 combines all of the samples of volunteer 1. In the same way, data of the faecal samples from volunteers 2, 3 and 4 were registered.

Figure 5 represents the resident bacterial biotypes detected in this study with four volunteers. This diagram shows the correlation between the number of a resident *Enterobacteriaceae* biotype and the

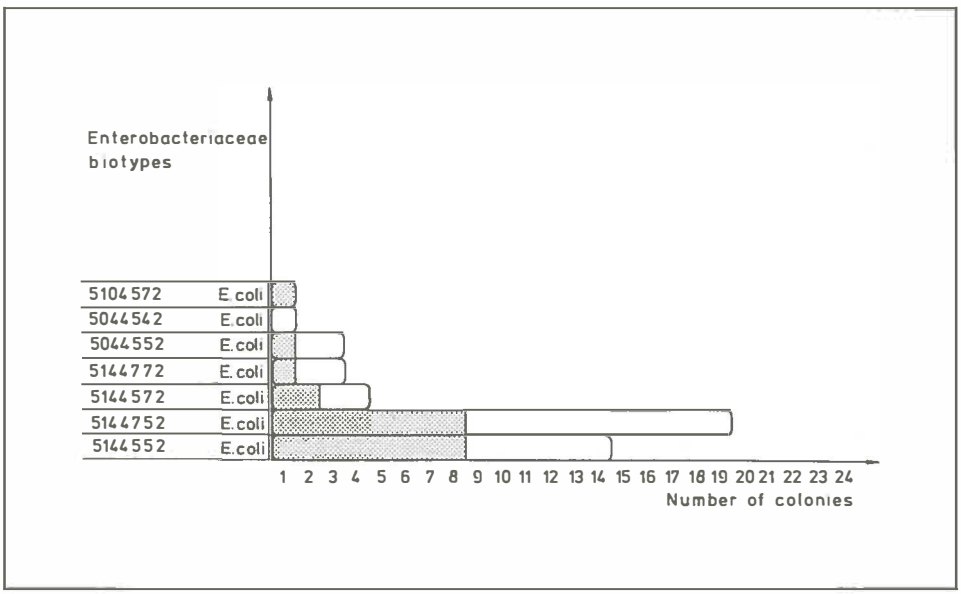


FIGURE 3

Inventory of faecal sample taken 24/3 (Figure 4). Number of different *Enterobacteriaceae* biotypes as a function of the number of colonies isolated in the slide. Number of IgA+ bacteria is indicated by shading on the abscissa.

number of IgA + representatives of the same biotype. The slope of the line is estimated by the mean of the fractions of coated *Enterobacteriaceae* calculated for all biotypes. The other line represents the 95 % confidence limit for the coated fractions. A total of 1388 *Enterobacteriaceae* colonies were isolated and identified; 1284 were considered as resident and 104 were recorded as transient. Seventeen resident biotypes were found, with an average of 75.5 colonies per resident biotype. The number of transient biotypes was 35, representing an average number of 2.9. Of the 1284 residents, 459 were IgA + ; 42 of the 104 transients were coated by IgA. This means a coating percentage of 34.9 % for the residents and 40.3 % for the transients.

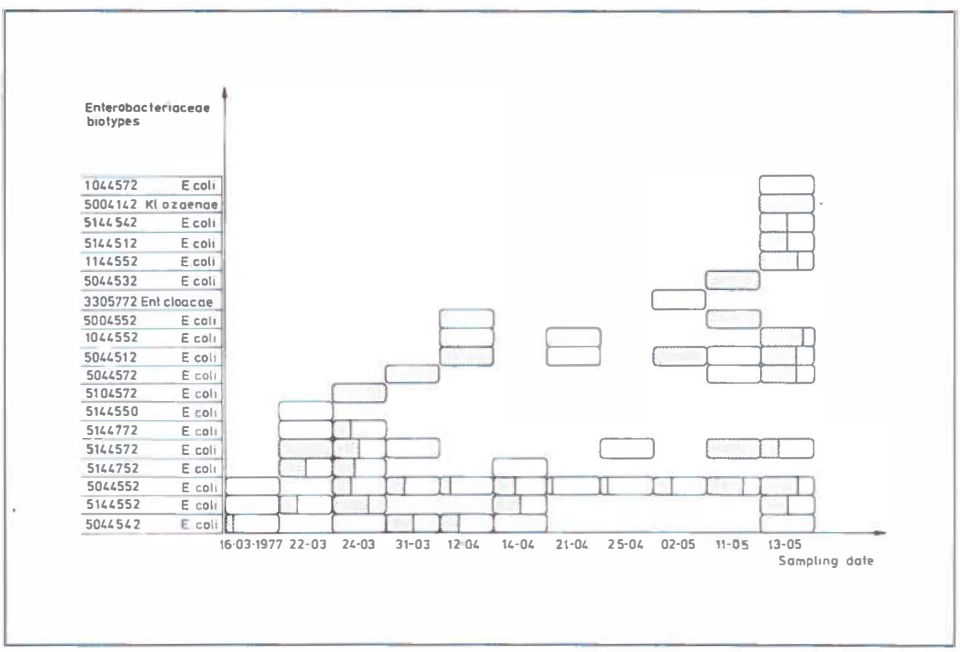


FIGURE 4
Colonization pattern of *Enterobacteriaceae* species of volunteer 1. All biotypes found during the entire sampling period are represented. The fraction of IgA + bacteria is again proportionally indicated by shading.

The fluorescence specificity was tested in two ways. After incubation of the conjugate with monoclonal IgA, IgM and IgG myeloma cells, there was fluorescence only with the IgA. Three cells were positive in the IgM system and one cell was fluorescent in the IgG system. Such results can be considered as normal. The conjugate dilution in which the fluorescence was optimal was 1 : 10. Several controls showed that, after culturing and reincubating with conjugate, the *Enterobacteriaceae* did not possess Fc receptors and that no antibacterial antibodies were present in the conjugate.

Repeated tests indicated that there was no difference between the number of *Enterobacteriaceae* colonies counted on the control plates of a treated fraction and the number of colonies of an untreated fraction of the same faecal sample. An optimal inventory of a faecal sample was usually obtained by picking 16-20 colonies from the slide.

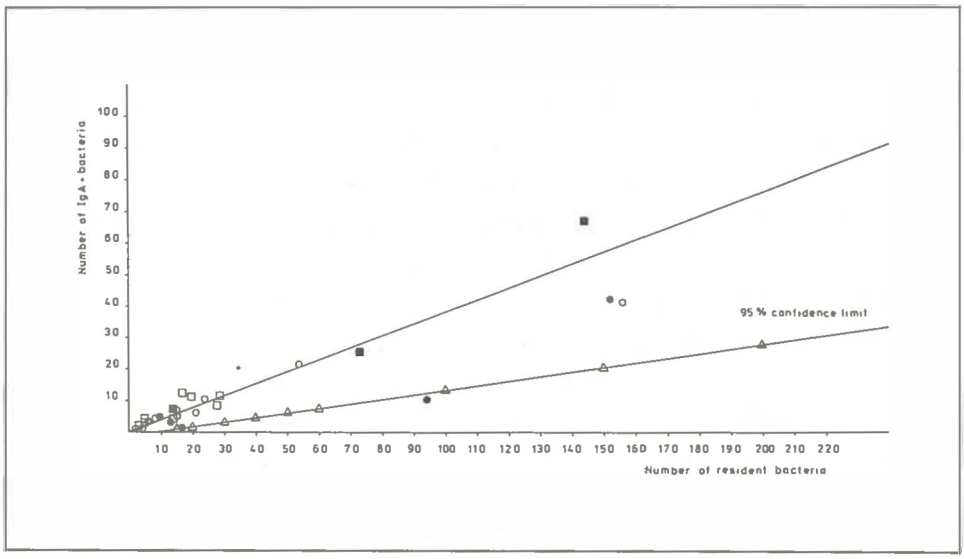


FIGURE 5
Diagram showing the resident *Enterobacteriaceae* biotypes detected in faeces of the four volunteers (○, volunteer 1; ●, volunteer 2; □, volunteer 3; ■, volunteer 4). Correlation between number of representatives of a particular resident biotype and number of IgA+ organisms of that biotype. Triangles indicate 95% confidence limit.

DISCUSSION

These experiments show that resident (colonizing) intestinal bacteria reaching high concentrations in the digestive tract are coated with IgA. This strengthens the hypothesis of Van der Waaij and Heidt (1977). In mice, resident bacteria at peak concentration were capable of penetrating the intestinal mucosa and these PPMs were subsequently cultured from the regional lymph nodes. This mechanism could explain stimulation of IgA producing cells.

The techniques used in these experiments only permitted detection and screening of bacterial biotypes and screening of bacterial biotypes at concentrations of $\geqslant 10^5$ bacteria per gram of faeces. All biotypes detected and examined were IgA coated. This 10^5 concentration is possibly also the threshold level which the intestinal bacteria have to exceed in order to penetrate the mucosa and to induce IgA production.

This selective effect explains the high incidence of resident bacteria (92 %). The threshold value could also explain the finding that there is no significant difference between the coating percentage of transient (40 %) and of resident (34 %) *Enterobacteriaceae.*

It is improbable that *Enterobacteriaceae* in the faeces are coated with IgM or IgG, since these two immunoglobulins are not protected against faecal proteolytic enzymes. IgA, however, is protected by a secretory fragment (Heremans, 1974). The presence of antibacterial antibodies in the conjugate suggests that the immunized animal, the goat, must have been infected by the corresponding bacterium, which seems improbable. Tests failed to demonstrate *Enterobacteriaceae* Fc receptors, which are reported only on gram-positive bacteria (Myhre and Kronvall, 1977).

In the present work, the API E 20 system, based on biochemical reactions, was used. *Enterobacteriaceae* species may also be typed serologically. There are two reasons why the biotyping system was preferred — its technical practicability and the relatively low cost. Moreover, the literature date indicate that biotypes run parallel to serotypes (Van der Waaij et al., 1975).

ACKNOWLEDGEMENTS

We are grateful to Dr. V. Fidler for statistical advice and to Mrs. M. Sikkens-De Zwaan for technical assistance. We wish to thank Prof. Dr. H. The for advice on immunological methods and for testing the conjugates.

REFERENCES

[1] HEREMANS, J.F. (1975). The secretory immune system. A critical appraisal. In: *The immune system and infectious diseases.* Karger, Basel, 376-385.

[2] GIBBONS, R.J. (1974). Bacterial adherence to mucosal surfaces and its inhibition by secretory antibiodies. In: *The immunoglobulin A system.* Eds. Mestecky, J., Lawton, A.R. Plenum Press, New York, 315-325.

[3] VAN DER WAAIJ, D., HEIDT, P. (1977). Intestinal bacterial ecology in relation to immunological factors and other defence mechanisms. In: *Food and Immunology.* Eds. Hambraeus, L., Hanson, L.A. Almqvist & Wiksell, Uppsala, 133-140.

[4] HEREMANS, J.F. (1974). Immunoglobulin A. In: *The Antigens.* Ed. Sela, M. Academic Press, London, 365-522.

[5] MYEROWITZ, R.L., ALBERS, A.C., YEE, R.B., ORSKOV, F. (1977). Relationship of K1 antigen to biotype in clinical isolates of *Escherichia coli. J.Clin.Microbiol.,*2:124-127.

[6] VAN DER WAAIJ, D., COHEN, B.J., ANVER, M.R. (1974). Mitigation of experimental inflammatory bowel disease in guinea pigs by selective elimination of the aerobic Gram-negative intestinal microflora. *Gastroenterology,* 67:640-472.

[7] MYHRE, E.B., KRONVALL, G. (1977). Heterogeneity of nonimmune immunoglobulin Fc reactivity among Gram-positive cocci: description of three major types of receptors for human immunoglobulin G. *Infect.Immun.,*17:475-482.

[8] VAN DER WAAIJ, D., SPELTIE, T.M., GUINEE, P.A.M., AGTERBERG, C. (1975). Serotyping and biotyping of 160 *Escherichia coli* strains: comparative study. *J.Clin.Microbiol.,*1:237-238.

II

IN VIVO BINDING OF SECRETORY IMMUNOGLOBULIN A TO NORMAL GASTROINTESTINAL FLORA

H.K.F. van Saene and D. van der Waaij.

Laboratory for Medical Microbiology,
University Hospital, Groningen, The Netherlands.

We find that on average, 40 % of the aerobic bacilli in the normal faeces are coated with secretory immunoglobulin A. This is presented as evidence for the role of gut associated lymphoid tissue in controlling normal gut flora by preventing adherence and colonization. Based on these findings for normal individuals, a case is made for decreased gut associated lymphoid tissue activity in those cases where less than 12.5 % of aerobes are coated.

INTRODUCTION

The gut associated lymphoid tissue (GALT) plays a key role in infection control and in preventing absorption of intestinal antigens (immune exclusion)[1]. Secretory immunoglobulin A (s-IgA) on the mucous membranes may be involved in this protective GALT activity by interfering with bacterial adherence. Attachment to mucosal surfaces via fimbriae is believed to be a prerequisite facilitating colonization and penetration of the digestive tract. Adherence of fimbriated bacteria is apparently inhibited by s-IgA shielding the fimbriae by coating bacteria[2]. s-IgA concentrations in serum and in external secre-

tions may less directly reflect GALT activity and this quantitative evidence may appear less important as a measure for the functional characteristics of s-IgA[3]. Therefore we studied the functional quality of GALT activity by determining its coating capacity. An *in vivo* test system incorporating a fluorescence immuno assay (FIA) technique, is used to evaluate the number of faecal bacteria coated with s-IgA[4].

SUBJECTS AND METHODS

GALT-FIA assay

In the present report GALT activity on faecal flora in normal man was studied by means of the GALT-FIA technique previously described[4].

1. Faecal flora

The faecal bacteria that we focused on in this assay were the aerobic *Enterobacteriaceae.* Other microorganisms such as staphylococci, streptococci and yeasts were not considered because they often give false positive staining with immunofluorescent anti-IgA. For, Gram-positive bacteria possess Fc receptors to which immunofluorescent anti human s-IgA molecules can adhere by their Fc part instead of binding with the Fab part of the molecule ('sticky' effect). Fc receptors are not detected on *Enterobacteriaceae*[5]. Secondly, *Enterobacteriaceae* can be typed in different ways. Typing is necessary in order to differentiate resident bacteria from transient ones. Biotyping system was preferred in this assay to serotyping, because of its technical practicability and the relative low costs. Moreover, the literature data indicate that biotypes run parallel to serotypes[6]. Comparing the total microscopic count with the numbers of *Enterobacteriaceae* that subsequently grew on the agar slide differentiation between the anaerobes and the aerobic *Enterobacteriaceae* could be made.

2. Techniques

In brief, a faecal suspension was stained by a conjugate, antihuman s-IgA labelled with FITC. This fluorescent suspension was

then placed on a MacConkey agar slide and examined by phase contrast and by immunofluorescence microscopy. The phase contrast examination showed anaerobes outnumbering aerobes by a factor of 10^4. When this field was then screened by immunofluorescence only s-IgA + (fluorescence +) bacteria were seen. The locations of s-IgA + bacteria which showed up as green citrons were then recorded. After microscopy the agar slide and its suspension cover was incubated at 37° C for 18 hours. Because s-IgA was not bactericidal colonies were obtained. Finally, the localization of the colonies was registered and the colonies identified and typed. By thus combining microscopic, culture and identification techniques we were able to establish which colonies were the progeny of s-IgA + bacteria. In typing we distinghuished between organisms which remained in the tract for more than one week (resident bacteria) from bacteria which appeared only sporadically in the duration of the eight week study (transient ones).

3. Controls

The fluorescence specificity was tested in two ways. After incubation of the conjugate with monoclonal IgA, IgM and IgG myeloma cells, there was fluorescence only with the IgA. After culturing and reincubating with conjugate, *Enterobacteriaceae* did not possess Fc receptors and controls showed that no antibacterial antibodies were present in the conjugate.

Concerning reproducibility, repeated tests indicated that there was no difference between the number of *Enterobacteriaceae* colonies of a treated fraction and the number of colonies of an untreated fraction of the same faecal sample.

Volunteers

Normal values for the percentage of s-IgA coated bacteria were determined, using the GALT-FIA technique, on stool specimens obtained from ten healthy volunteers. Specimens were collected thrice weekly for approximately 8 weeks.

From the 116 stool samples (an average of 11.6 per volunteer), a total of 2003 *Enterobacteriaceae* colonies were isolated, identified and typed; 1522 were found to be resident (76 %) and 481 were recorded as transient (24 %). From the 1522 resident *Escherichia coli* bacteria, 581 were s-IgA coated (38.5 %). One hundred seventy two of the 481 transients were coated with s-IgA, corresponding to a coating percentage of 37.7 %. Figure 2 represents the 25 resident *Escherichia coli* types detected in the 116 stool samples, and shows the correlation between the number of isolated colonies of a particular resident *Escherichia coli* and the number of s-IgA coated representatives of that same resident *Escherichia coli.* The slope of the line is estimated by the mean of the fractions of coated bacteria calculated for all resident resident *Escherichia coli* types. The dotted line represents the 95 % confidence

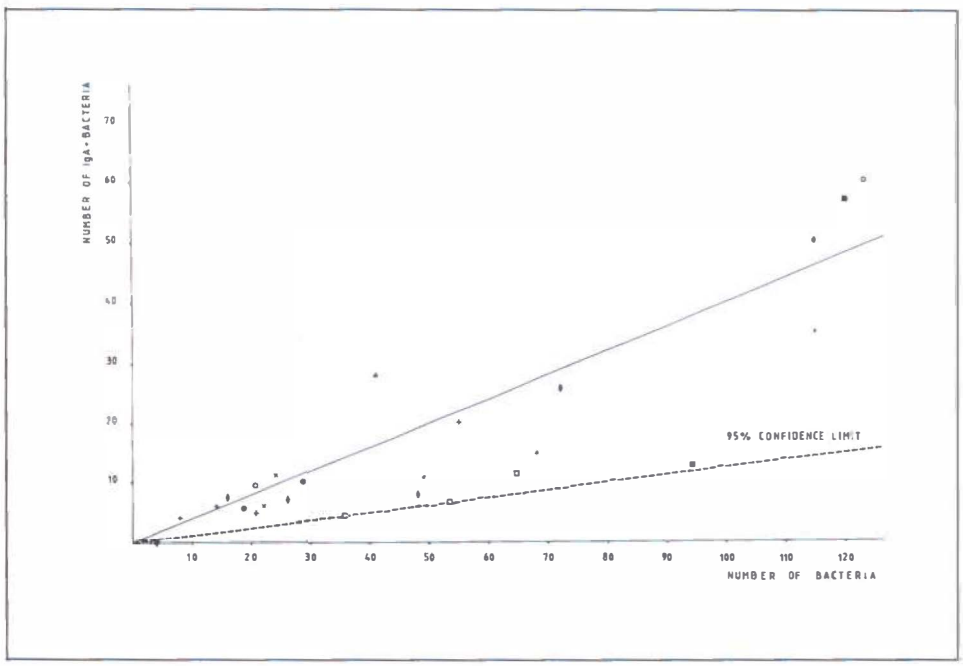


FIGURE 2
Enterobacteriaceae resident types detected in 116 stool samples from 10 volunteers (○: volunteer 1, ●: volunteer 2 etc.). Correlation between the number of representatives of a particular resident *E. coli* type and the number of s-IgA positive organisms of that resident *E. coli* strain. Dotted line indicates 95 % confidence limit.

lower limit for the s-IgA coated *Escherichia coli* fractions. According to these statistics a percentage of about 40 can be considered as 'normal', while less than 12.5 % can be interpreted as a significant reduction.

In contrast to the aerobic *Enterobacteriaceae,* the far more numerous anaerobes were not s-IgA coated. All s-IgA+ (fluorescence+) bacteria gave rise to (aerobic) colonies after incubation.

DISCUSSION

There is a considerable body of evidence for local response to normal endogenous flora[7,8]. This study shows that in faeces of healthy individuals, only aerobic bacteria are coated with s-IgA. The endogenous anaerobes are apparently not able to stimulate the secretory immune system even though they colonize the intestines in very high numbers. This may be due to their low degree of immunogenicity[9,10]. The present study on humans confirm earlier work on mice by van der Waaij who found that under normal conditions aerobic bacteria could invade the mucosa and be recovered in the lymphatic organs when they colonize the gut in concentrations normally observed for anaerobes[11].This mechanism could explain stimulation of s-IgA-producing cells by aerobic bacteria.

That only a few *E. coli* types predominate as residents in normal stools is a well known phenomenon[12,13]. The GALT — by interacting only with the aerobic part of the endogenous microflora — seems to function as a regulating (s-IgA coating) apparatus preventing adherence, excessive growth and possible subsequent invasion by *E. coli* and other *Enterobacteriaceae.* The coating phenomenon of aerobic flora (both resident and transient) might also be related to stimulation of GALT by antigens supplied by food[11,13].

A coating percentage of 40 could be considered as 'normal'. The fact that s-IgA is present mainly on the mucous layer covering the gut mucosa may explain our finding that not all cells of an aerobic bacterium are coated with s-IgA. Bacteria passing near the mucous

membranes (mucosal-associated flora) have a greater chance of being coated than organisms confined to the centre of the gut lumen (luminal-associated flora) where the concentration of s-IgA molecules is not as high[14]. In addition, s-IgA is not bactericidal so that bacteria still capable of multiplying during intestinal passage may lose their s-IgA coat as a result of cell division[15].

Our findings that the GALT activity is 'reduced' when the number of s-IgA coated endogenous *E. coli* is lower than 12.5 % suggests clinical relevance. Reduced s-IgA coating might be related to an increased permeability of the intestinal tract leading to formation of antibodies. In this regard GALT-FIA technique — as a functional test for local immunity — could be of value in the diagnosis of important clinical disorders such as selective IgA deficiency, auto-immunity and graft-versus-host disease[16,17,18].

ACKNOWLEDGEMENTS

We are grateful to Mrs. E. van Santen and Mr. J. Krikken for technical, and to Mrs. S. Swart for secretarial assistance. We also wish to thank Dr. V. Fidler for suggestions regarding the statistical analysis.

REFERENCES

[1] WALKER, W.A., ISSELBACHER, K.J. (1977). Intestinal antibodies. *New Engl. J.Med.*,297:767-773.

[2] BERKELEY, R.C.W., LYNCH, J.M., MELLING, J., RUTTER, P.R., VINCENT, B. (1980). *Microbial adherence to surfaces.* Ellis Horwood, London.

[3] TOMASI, T.B. Jr., LARSON, L., CHALLACOMBE, S., Mac NABB, P. (1980). Mucosal immunity: the origin and migration patterns of cells in the secretion system. *J.Allergy Clin.Immunol.*,65:12-19.

[4] VAN SAENE, H.K.F., VAN DER WAAIJ, D. (1979). A novel technique for detecting s-IgA coated potentially pathogenic microorganisms in the human intestine. *J.Immunol.Methods,*30:87-96.

[5] MYHRE, E.B., KRONVALL, G. (1977). Heterogeneity of nonimmune immunoglobulin Fc reactivity among Gram-positive cocci: description of three major types of receptors for human immunoglobulin G. *Infect.Immun.*,17:475-482.

[6] VAN DER WAAIJ, D., SPELTIE, T.M., GUINEE, P.A.M., AGTERBERG, C. (1975). Serotyping and biotyping of 160 *Escherichia coli* strains: comparative study. *J.Clin.Microbiol.*,1:237-238.

[7] BRANDTZAEG, P., FJELLANGER, J., GJERSIELDEN, S.T. (1968). Adsorption of immunoglobulin A onto oral bacteria in vivo. *J.Bacteriol.*,96:242-249.

[8] SHEARMAN, D.J.C., PARKIN, D.M., McCLELLAND, D.B.L. (1972). The demonstration and function of antibodies in the gastrointestinal tract. *Gut*, 13:483-499.

[9] FOO, M.C., LEE, A. (1972). Immunological response of mice to members of the autochtonous intestinal microflora. *Infect.Immun.*,6:525-532.

[10] BERG, R.D., SAVAGE, D.C. (1972). Immunological responses and microorganisms indigenous to the intestinal tract. *Am.J.Clin.Nutr.*,25:1364-1371.

[11] VAN DER WAAIJ, D., HEIDT, P. (1977). Intestinal bacterial ecology in relation to immunological factors and other defence mechanisms. In: *Food and Immunology*. Eds. Hambraeus, L., Hanson, L.A. Almqvist & Wiksell, Uppsala, 133-140.

[12] SHOOTER, R.A., BETTELHEIM, K.A., LENNOX-KING, S.M.J., O'FARRELL, S. (1977). *Escherichia coli* serotypes in the faeces of healthy adults over a period of several months. *J.Hyg.*,78:95-98.

[13] BETTELHEIM, K.A., FAIERS, M., SHOOTER, R.A. (1972). Serotypes of *Escherichia coli* in normal stools. *Lancet*,ii:1224-1226.

[14] CRABBE, P.A., BAZIN, H., EYSSEN, H., HEREMANS, J.F. (1968). The normal microbial flora as a major stimulus for proliferation of plasma cells synthesizing IgA in the gut. *Int.Arch.Allergy.*,34:362-375.

[15] HEREMANS, J.F. (1975). The secretory immune system. A critical appraisal. In: *The immune system and infectious diseases.* Karger, Basel,376-385.

[16] AMMANN, A.J., HONG, R. (1970). Selective IgA deficiency and auto-immunity. *Clin.Exp.Immunol.*,7:833-838.

[17] CUNNINGHAM-RUNDLES, C., BRANDEIS, W.E., SAFAI, B., O'REILLY, R., DAY, N.K., GOOD, R.A. (1979). Selective IgA deficiency and circulating immune complexes containing bovine proteins in a child with chronic graft-versus-host disease. *Am.J.Med.*,67:883-890.

[18] BESHORNER, W.E., YARDLEY, J.H., TUTSCHKA, P.J., SANTOS, G.W. (1981). Deficiency of intestinal immunity with graft-versus-host disease in humans. *J.Inf.Dis.*,144:38-46.

III

EVIDENCE FOR DYSFUNCTION OF GUT ASSOCIATED LYMPHOID TISSUE IN INFLAMMATORY BOWEL DISEASE PATIENTS

H.K.F. van Saene[1], W. Jansen[2], V. Fidler[3], W.C. Veeger[4] and D. van der Waaij[5].

Laboratory for Medical Microbiology[1,5],
Department of Medical Statistics[3]
and Department of Gastroenterology[2,4].
University Hospital, Groningen, The Netherlands.

The activity of gut associated lymphoid tissue (GALT) in 20 patients with inflammatory bowel disease (IBD) (10 with Crohn's disease and 10 with ulcerative colitis) was compared with a control group of healthy individuals. The GALT function measured in this study was the coating of intestinal bacteria with secretory immunoglobulin A (s-IgA). In all patients a statistically significant reduction of the percentage of bacteria coated with s-IgA was found. These findings indicate a dysfunction of GALT suggesting a mucosal immunodeficiency. Whether these observations imply a secretory component disorder or whether they are due to abnormalities of T-cell function, is discussed.

INTRODUCTION

The gut associated lymphoid tissue (GALT) plays a key role in controlling normal gut flora by preventing adherence and colonization[1]. Secretory immunoglobulin A (s-IgA) on the mucous membranes may be involved in this controlling GALT activity by interfering with

bacterial adherence. Attachment to mucosal surfaces via fimbriae is believed to be a prerequisite facilitating colonization (and subsequent translocation) of intestinal bacteria (or antigens). Adherence of fimbriated bacteria is apparently inhibited by s-IgA shielding the fimbriae by coating bacteria[2]. s-IgA concentrations in serum and in external secretions may less directly reflect GALT activity and this quantitative evidence may appear less important as a measure for the functional characteristics of s-IgA[3]. Therefore we studied the functional quality of GALT activity by determining its coating capacity. An *in vivo* test system incorporating a fluorescence immuno assay (FIA) technique, is used to evaluate the number of faecal bacteria coated with s-IgA[4].

The diseases known collectively as inflammatory bowel diseases (IBDs) are ulcerative colitis (UC) and Crohn's disease (CD). Their aetiopathogenesis remains unknown. There is considerable evidence for the involvement of an auto-immunological process[5]. One possibility is an altered immune reactivity to digestive tract-associated antigens, including enterobacterial antigens[6]. This hypothesis implies a state of hypersensitivity to intestinal antigens. Development of hypersensitivity is depending on translocation of bacteria (or bacterial antigens) from the digestive tract into the lymphoid organs (e.g., mesenteric lymph nodes, spleen)[7]. High intestinal concentrations of bacteria are — at least in experimental animals — associated with translocation[8]. Increased translocation is very likely to be followed by increased titres of circulating immunoglobulins[9]. Translocation is possibly facilitated by a deficiency of GALT as has been found in nude mice[10].

It is in the light of these considerations that we decided to study the incidence of IgA coated bacteria, i.e., the coating capacity of the gut associated lymphoid tissue in inflammatory bowel disease patients and in healthy controls.

SUBJECTS AND METHODS

GALT-FIA assay

In the present report GALT activity on faecal flora in twenty pa-

tients with inflammatory bowel disease and in normal individuals was studied by means of the GALT-FIA technique previously described[4].

1. Faecal flora

The faecal bacteria that we focused on in this assay were the aerobic *Enterobacteriaceae*. Other microorganisms such as staphylococci, streptococci and yeasts were not considered because they often give false positive staining with immunofluorescent anti-IgA. For, Gram-positive bacteria possess Fc receptors to which immunofluorescent anti human s-IgA molecules can adhere by their Fc part instead of binding with the Fab part of the molecule ('sticky' effect). Fc receptors are not detected on *Enterobacteriaceae*[11]. Secondly, *Enterobacteriaceae* can be typed in different ways. Typing of bacteria isolated from a subject involved in this study was necessary in order to differentiate resident bacteria from transient ones. Biotyping[12] was preferred in this assay to serotyping, because of its technical practicability and the relative low costs. Moreover, the literature date indicate that biotypes often run parallel to serotypes[13,14]. Comparing the total microscopic count with the numbers of *Enterobacteriaceae* that subsequently grew on the agar slide differentiation between the anaerobes and the aerobic *Enterobacteriaceae* could be made.

2. Techniques

In brief, a faecal suspension was stained by a conjugate, anti human s-IgA labelled with FITC. This fluorescent suspension was then placed on a MacConkey agar slide and examined by phase contrast and by immunofluorescence microscopy. The count under phase contrast outnumbered the microorganisms growing on MacConkey agar by a factor of 10^4. When this field was then screened by immunofluorescence only s-IgA+ (fluorescence+) bacteria were seen. The locations of s-IgA+ bacteria which showed up as rods with a dense green lining were then recorded. After microscopy the agar slide and its suspension cover was incubated at 37° C for 18 hours. Because s-IgA is not bactericidal colonies were obtained. Finally, the localization of the colonies was registered and the colonies identified and typed. By thus combining microscopic, culture and identification techniques we were able to establish which colonies were very likely the progeny of s-IgA coated bacteria. In typing we could distinguish

between organisms which remained in the tract for more than one week, e.g., which were found in several subsequent samples (resident bacteria) from bacteria which were only sporadically isolated during the eight weeks of study (transient ones)[15].

3. Controls

The fluorescence specificity was tested in two ways. After incubation of the conjugate with monoclonal IgA, IgM and IgG myeloma cells, there was fluorescence only with the IgA. After culturing and reincubating with conjugate, *Enterobacteriaceae* did not possess Fc receptors.

A possible impairment of *Enterobacteriaceae* survival by the assay procedures was repeatedly controlled. No difference was found between the number of *Enterobacteriaceae* colonies of a fraction processed for the assay and the number of colonies of an unprocessed fraction of the same faecal sample.

Patients

The patients included in this study were twenty in-patients (ten with ulcerative colitis and ten with Crohn's disease) in the gastroenterology department of the University Hospital. All received sulfasalazine; none of the patients was treated with steroids, immunosuppressive or anti-diarrhoeal drugs and no member of the patient group underwent surgery before or during the period of study. Out of these 20 patients, fourteen were involved in a follow-up study when their disease was in complete remission. This remission group served as their own controls. For, in the relapse phase diarrhoea was a prominent characteristic causing a dilution effect possibly resulting in a lower s-IgA coating incidence. To avoid this possible artefact, the same patients — but now in remission phase — producing faeces of normal consistence were followed up.

Volunteers

A group of ten healthy laboratory co-workers served as control group. No member of the control group had experienced any intestinal illness.

Specimens

Faecal samples were collected thrice a week and investigated within one hour after defaecation. If immediate examination was not possible, the samples were frozen in liquid nitrogen.

Statistics

Statistical analysis was done by Fishers exact test.

RESULTS

Faecal flora

1. Volunteers

From the 116 stool samples (an average of 11.6 per volunteer) a total of 2003 *Enterobacteriaceae* colonies were isolated, identified and typed. The resident flora included *E. coli* only. Although 25 types were isolated, only two types of *E. coli* were predominant: bioprofiles 5144572 and 5044552. The transient flora however consisted not only of *Escherichia coli* but also of *Klebsiella* spp, *Enterobacter* spp, *Citrobacter* spp etc. Practically all representatives of the *Enterobacteriaceae* family were isolated as transients on single occasions in the stools of these healthy individuals. The number of transient strains was large: 102.

2. Patients

Relapse phase. From 202 stool samples (10.1 sample per patient) a total of 1685 *Enterobacteriaceae* colonies were isolated, identified and typed. No significant difference between the patient and the control group was found. A manifest but not significant difference was perhaps seen in the lower number of different resident *E. coli* strains in the patient group. In the ten healthy individuals there were 25 resident strains (2.5 strain per individual). A lower number of only 27 resident *E. coli* strains were detected in the faeces of the 20 IBD patients (1.3 strain per patient). The predominant bioprofiles however were identical. The number of transient strains was also relatively smaller (111) in the IBD group than in the control subjects.

Remission phase. A total of 74 samples (3.1 sample per patient) could be evaluated. In these samples an identical colonization pattern showing no major differences was observed.

Coating incidence

1. Volunteers

We found that on average, 40 % of the resident *E. coli* strains in the normal faeces were coated with s-IgA. Based on these findings in normal individuals, a case was made for decreased GALT activity in those patients in whom less than 12.5 % of aerobic resident *E. coli* were found to be coated[16] (Figure 1).

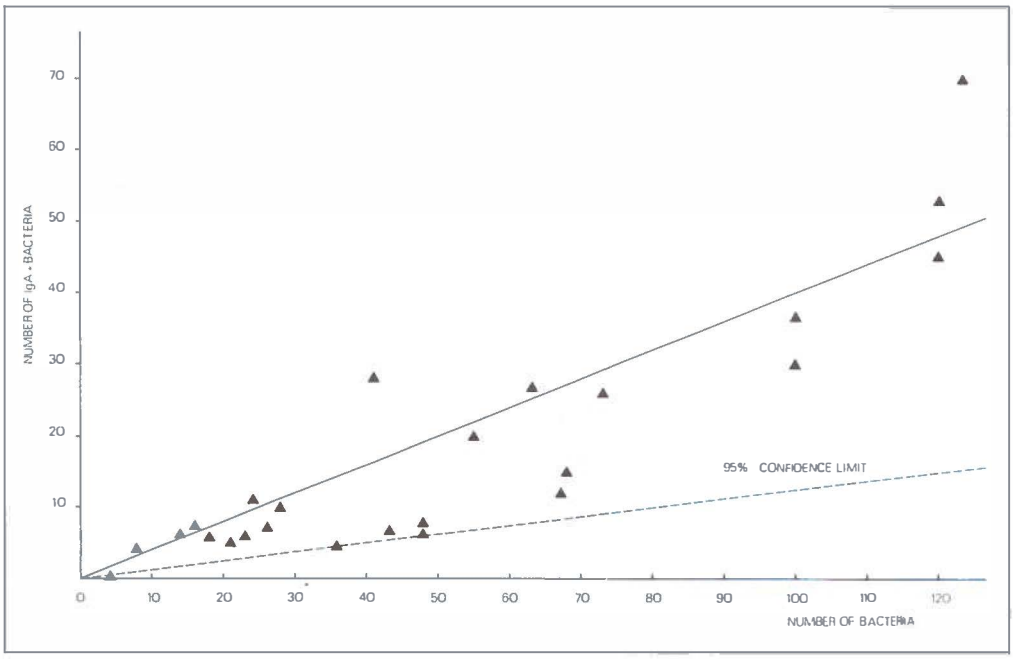


FIGURE 1

Twenty five resident *E. coli* strains isolated from faeces in healthy individuals. Correlation between the number of bacteria of a resident *E. coli* strain and the number of s-IgA positive microorganisms of that resident *E. coli* strain. On an average, 40 % is coated. Dotted line indicates 95 % confidence limit representing significant decreased GALT activity under this 12.5 % limit.

2. Patients

Relapse phase: from the 1193 resident *E. coli* strains no more than 69 were coated with s-IgA (6%). In all twenty IBD patients the coating incidence for all strains was found to be below the 95% confidence underlimit of 12.5% (Fishers exact test, $P < 0.0001$) (Figure 2). No differences were observed between the patients with ulcerative colitis and the patients with Crohn's disease.

Remission phase: from the 150 resident *E. coli* strains isolated from the 74 samples only 11 were s-IgA coated (7%).

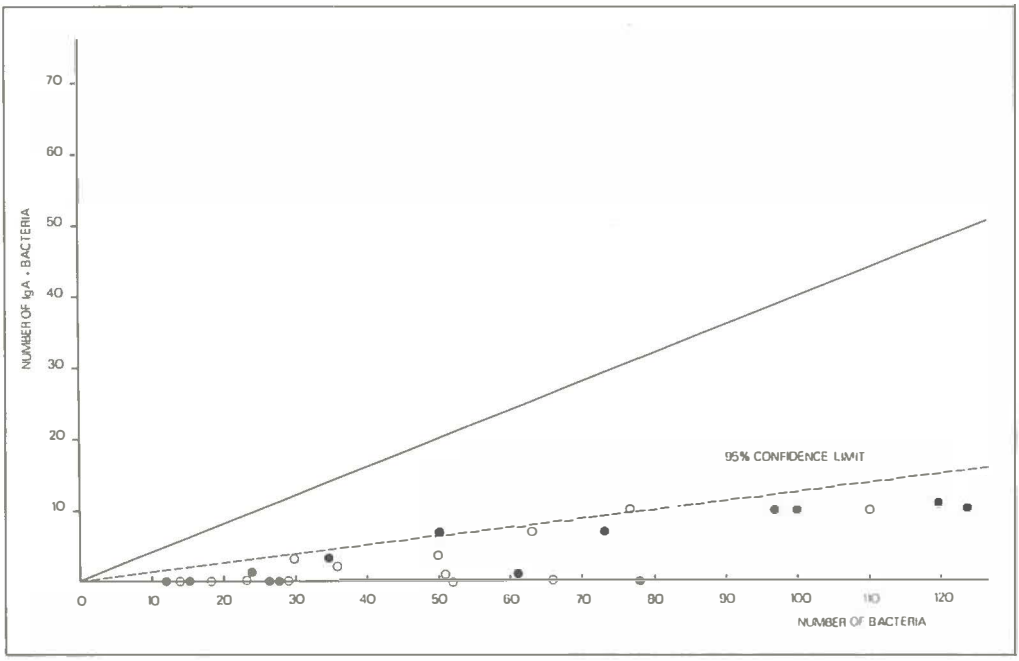


FIGURE 2

Twenty seven resident *E. coli* strains detected in stool samples from IBD patients (○: Crohn's disease, ●: ulcerative colitis). Ratio between the number of bacteria of a particular resident *E. coli* strain and the number of s-IgA + ones of that *E. coli* strain. In all patients the coating incidence was found to be underneath the 95% confidence underlimit of 12.5%.

DISCUSSION

In this study we made two important observations. (i) The faecal flora of IBD patients does not qualitatively and quantitatively differ from healthy controls as far as aerobic bacteria are concerned, neither when the disease is in relapse nor in remission. These results confirm earlier studies[17,18]. The low number of different *Enterobacteriaceae* spp in the patient group could be explained by the fact that all patients receive identical hospital food representing only a few different strains. (ii) Both in relapse as well as in remission phase IBD patients demonstrate a significant decreased IgA-coating incidence in Gram-negative aerobic bacteria. These observations indicate a decreased function of GALT: in both phases of the disease all patients show a mucosal immunodeficiency.

A deficiency of gut associated lymphoid tissue in IBD patients has earlier been described by others[19,20,21]. In one recent study the free secretory component of salivary s-IgA was absent or markedly diminished in several members of a family who had ulcerative colitis and in some unrelated patients with ulcerative colitis, suggesting a possible compromise in mucosal defences in IBD patients[22]. In an other study in patients with IBD peripheral-blood mononuclear cells showed increased synthesis and secretion of IgM, IgG and IgA, however intestinal mononuclear cells showed decreased antibody secretion[23]. These observations made in different centers with different methods fit very well with the impaired coating function of GALT found in the present study. All these findings reveal one common denominator: an immunodeficiency at mucosal site.

Secretory IgA-production appears to be T-cell dependent[24,25]. There are T-cells specifically concerned with helping and suppressing antibody production. The gut associated lymphoid tissue contains IgA helper cells but it is also a haven for IgG and IgM suppressor cells[26]. Oral antigen exposure would be expected not only to stimulate a brisk IgA response but also simultaneously to suppress the appearance of antibody of other classes[27,28]. The T-cell response to an oral antigen (e.g., translocating bacterial antigens) seems to have two important functions. It aids the formation of s-IgA antibody which acts locally

to limit adherence, and therewith colonization and subsequent translocation from the digestive tract. And by suppressing the formation of circulating antibody classes it ensures that the small quantities of antigen which are translocated across the mucosa do not provoke possible inflammation responses. A defect in the way that T-cells perform these tasks may underlie the altered immune responsiveness characteristic of inflammatory bowel disease[29]. Whether decreased GALT function is due to a decreased T-helper cell function or decreased s-IgA coating of bacteria is due to a secretory component deficiency, remains an open question. However, decreased coating of *Enterobacteriaceae* spp could be associated with enhanced adherence of *Enterobacteriaceae* spp to digestive tract mucosa[30]. Enhanced adherence of intestinal bacteria in general leads into high concentrations in faeces ($\geqslant 10^6$ per gram of faeces)[31]. This was not found in our patients nor in those of others as mentioned above. The normal concentrations of the aerobic *Enterobacteriaceae* spp make it very unlikely that increased translocation of *Enterobacteriaceae* spp has been of primary importance in the pathogenesis.

ACKNOWLEDGEMENTS

We thank the nursing staff and the volunteers for their collaboration and we express our appreciation to Mrs. E. van Santen and Mr. J. Krikken for technical assistance and to Mrs. M.H. Brummer-Scholten for secretarial assistance.

REFERENCES

[1] HEREMANS, J.F. (1975). The secretory immune system. A critical appraisal. In: *The immune system and infectious diseases.* Karger, Basel, 376-385.

[2] WILLIAMS, R.C., GIBBONS, R.J. (1972). Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. *Science,* 1977:697-699.

[3] TOMASI, T.B. Jr., LARSON, L., CHALLACOMBE, S., MacNABB, P. (1980). Mucosal immunity: the origin and migration patterns of cells in the secretion system. *J.Allergy Clin. Immunol.,* 65:12-19.

[4] VAN SAENE, H.K.F., VAN DER WAAIJ, D. (1979). A novel technique for detecting s-IgA coated potentially pathogenic microorganisms in the human intestine. *J.Immunol.Methods,* 30:87-96.

[5] JEWELL, D.P., HODGSON, H.J.F. (1976). Autoimmune and inflammatory diseases of the gastrointestinal tract. In: *Immunological aspects of the liver and gastrointestinal tract.* Eds. Ferguson, A., Mac Sween, R.N.M. Medical and Technical Press, Lancaster, 203-250.

[6] SHORTER, R.G., HUIZENGA, K.A., SPENCER, R.J. (1972). A working hypothesis for the aetiology and pathogenesis of non-specific inflammatory bowel disease. *Digestive Diseases,* 17:1024-1032.

[7] BERG, R.D. (1979). Ph. D. thesis.

[8] BERG, R.D., SAVAGE, D.C. (1975). Immune responses of specific pathogen-free and gnotobiotic mice to antigens of indigenous and nonindigenous microorganisms. *Infect.Immun.,* 11:320-329.

[9] WRIGHT, R., TRUELOVE, S.C. (1966). Auto-immune reactions in ulcerative colitis. *Gut,* 7:32-40.

[10] OWENS, W.E., BERG, R.D. (1980). Bacterial translocation from the gastrointestinal tract of athymic (nu/nu) mice. *Infect.Immun.,* 27:461-467.

[11] MYHRE, E.B., KRONVALL, G. (1977). Heterogeneity of nonimmune immunoglobulin Fc reactivity among Gram-positive cocci: description of three major types of receptors for human immunoglobulin G. *Infect.Immun.,* 17:475-482.

[12] SMITH, P.B., TOMFOHRDE, K.M., RHODEN, D.L., BALOWS, A. (1972). API system: a multitube micromethod for identification of *Enterobacteriaceae. Appl.Microbiol.,* 24:449-452.

[13] BETTELHEIM, K.A., TAYLOR, J. (1969). A study of *Escherichia coli* isolated from chronic urinary infection. *J.Med.Microbiol.,* 2:225-236.

[14] VAN DER WAAIJ, D., SPELTIE, T.M., GUINEE, P.A.M., AGTERBERG, C. (1975). Serotyping and biotyping of 160 *Escherichia coli* strains: comparative study. *J.Clin.Microbiol.,* 1:237-238.

[15] ROBINET, H.G. (1962). Relationship of host antibody to fluctuations of *Escherichia coli* serotypes of human intestine. *J.Bacteriol.,* 84:896-901.

[16] VAN SAENE, H.K.F., VAN DER WAAIJ, D. (1982). *In vivo* binding of secretory immunoglobulin A to normal gastrointestinal flora. *Gut,* in press.

[17] SENECA, H., HENDERSON, E. (1950). Normal intestinal bacteria in ulcerative colitis. *Gastroenterology,* 15:34-39.

[18] WEINSTEIN, L. (1961). Bacteriologic aspects of ulcerative colitis. *Gastroenterology,* 4:323-330.

[19] GREEN, F.H.Y., FOX, H. (1975). The distribution of mucosal antibodies in the bowel of patients with Crohn's disease. *Gut,* 16:125-131.

[20] GELZAYD, E.A., KRAFT, S.C., FITCH, F.W., KIRSNER, J.B. (1968). Distribution of immunoglobulins in human rectal mucosa. II. Ulcerative colitis and abnormal mucosal control subjects. *Gastroenterology,* 54:341-347.

[21] DAS, K.M., ERBER, W.F., RUBINSTEIN, A. (1977). Immunohistochemical changes in morphologically involved and uninvolved colonic mucosa of patients with idiopathic proctitis. *J.Clin.Invest.,* 59:379-385.

[22] ENGSTROM, J.F., ARVANITAKIS, C., SAGAWA, A., ABDOU, N.I. (1978). Secretory immunoglobulin deficiency in a family with inflammatory bowel disease. *Gastroenterology,* 74:747-751.

[23] MacDERMOTT, R.P., NASH, G.S., BERTOVICH, M.J., SEIDEN, M.V., BRAGDON, M.J., BEALE, M.G. (1981). Alterations of IgM, IgG and IgA synthesis and secretion by peripheral blood and intestinal mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Gastroenterology,* 81:844-852.

[24] PRITCHARD, H., RIDDAWAY, J., MICKLEM, H.S. (1973). Immune responses in congenitally thymus-less mice. II. Quantitative studies of serum immunoglobulins, the antibody response to sheep erythrocytes, and the effect of thymus allografting. *Clin.Exp.Immunol.,* 13:125-138

[25] GUY-GRAND, D., GRISCELLI, C., VASSALLI, P. (1975). Peyer's patches, gut IgA plasma cells and thymic function: study in nude mice bearing thymic grafts. *J.Immunol.,* 115:361-364.

[26] ELSON, C.O., HECK, J.A., STROBER, W. (1979). T-cell regulation of murine IgA synthesis. *J.Exp.Med.,* 149:632-643

[27] SWARBRICK, E.T., STOKES, C.R., SOOTHILL, J.F. (1979). Absorption of antigens after oral immunisaton and the simultaneous induction of specific systemic tolerance. *Gut,* 20:121-125.

[28] STOKES, C.R., SWARBRICK, E.T., SOOTHILL, J.F. (1980). Immune elimination and enhanced antibody responses: functions of circulating IgA. *Immunology,* 40:455-458.

[29] LANCET (1979). Editorial. Gut reaction to antigen. *Lancet,* i:763.

[30] VAN DER WAAIJ, D., HEIDT, P.J. (1979). Intestinal bacterial ecology in relation to immunological factors and other defence mechanisms. In: *Food and Immunology.* Eds. Hambraeus, L., Hanson, L.A. Almqvist & Wiksell, Stockholm, 133-141.

[31] GIBBONS, R.J. (1974). Bacterial adherence to mucosal surfaces and its inhibition by secretory antibodies. In: *The immunoglobulin A system.* Eds. Mestecky, J., Lawton, A.R. Plenum Press, New York, 315-325.

CHAPTER FIVE

SYSTEMIC IMMUNE SYSTEM

„l'anaphylaxie signifie le contraire de la protection"

Richet, C.

PREVALENCE OF ENDOGENOUS ENTEROBACTERIACEAE AND OF CIRCULATING ANTIBODIES TO ENDOGENOUS ENTEROBACTERIACEAE IN INFLAMMATORY BOWEL DISEASE PATIENTS

H.K.F. van Saene[1], W. Jansen[2], V. Fidler[3], W.C. Veeger[4] and D. van der Waaij[5]

Laboratory for Medical Microbiology[1, 5]
Department of Medical Statistics[3] and Department of Gastroenterology[2, 4],
University Hospital, Groningen, The Netherlands.

Sera from 20 patients with inflammatory bowel disease (IBD) (10 with Crohn's disease and 10 with ulcerative colitis) were assayed for the presence of circulating antibodies against endogenous Enterobacteriaceae spp and were compared with sera from 10 healthy control subjects. The findings indicate that the majority of IBD patients were more immunologically responsive to antigens of resident E. coli strains than did the healthy subjects. For, titres of IgG and of IgA class were significantly increased (<0.025). The two possibilities — circulating antibodies to endogenous resident E. coli are secondary to increased translocation or to a decrease of T-cell suppressor activity — are discussed.

INTRODUCTION

The diseases collectively known as inflammatory bowel diseases (IBDs) are ulcerative colitis (UC) and Crohn's disease (CD). The pathogenesis remains unknown. There is evidence that an autoimmunological process may be involved. In this respect an altered immune reactivity to digestive tract-associated antigens, including

enterobacterial antigens is to be considered[1, 2]. In this investigation we focus on the *Enterobacteriaceae* microorganisms (or enterobacterial antigens) for the following reasons: (i) in normal circumstances aerobic *Enterobacteriaceae* are more invasive than the anaerobes, notwithstanding the fact that the anaerobic microflora outnumbers the aerobes more than 10^4 times[3, 4]. (ii) The local immunity of the digestive tract (s-IgA) only reacts with aerobic microorganisms by coating them. Anaerobes are never coated by s-IgA. The predominating endogenous anaerobes are not able to stimulate the secretory immune system even though they colonize the intestines in very high numbers[5, 6]. If the hypothesis that IBDs are expression forms of immune reactivity against digestive tract-associated antigens is valid, we assume that the aerobic *Enterobacteriaceae* are the most likely candidates for translocation, i.e., passage from the digestive tract lumen through the mucosal barrier into the systemic immune system. In (genetically?) predisposed individuals with a decreased function of gut associated lymphoid tissue (GALT), translocation of bacterial antigens may occur and result in stimulation of lymphoid organs, e.g., mesenteric lymph nodes, spleen. *Enterobacteriaceae* microorganisms sharing antigens with host constituents may induce cross-reacting antibodies, i.e., antibodies which also complex with host tissue components[7]. Finally, these cross-reacting antibodies could mediate in the immunological inflammation reactions (type 2 or 3) and/or granuloma-formation.

In this investigation, we have adressed the issue of whether IBD patients are hypersensitized against their own digestive tract flora (enterobacterial antigens) versus healthy subjects without intestinal disease. We have investigated the prevalence of antibodies to endogenous *Enterobacteriaceae* (or antigens) recovered from the faeces of each individual by means of an indirect immunofluorescent assay (IFA).

SUBJECTS AND METHODS

Subjects

The 20 patients investigated (10 with Crohn's disease and 10 with ulcerative colitis) were adults for which prolonged hospitalization seemed likely at the time of their admission to the gastroenterology department of the University Hospital. No patient selected for the study had been given antimicrobial agents (AMAs) or immunosuppressive drugs in the month prior to hospitalization nor during the 6 weeks of bacteriological and serological monitoring.

Ten laboratory co-workers served as a control group. None of the control individuals had experienced acute illness or received any antimicrobial therapy during the study or in the month preceding it.

Studies on faecal flora (Enterobacteriaceae)

Specimens of faeces were obtained twice weekly for six consecutive weeks. Each faecal specimen was processed within 30 minutes of being passed. Concentrations of the aerobic *Enterobacteriaceae* were determined as follows: one gram of faeces was homogenized in 9 ml of brain heart infusion broth (Oxoid), serially diluted (1:10) and incubated for 18 hours at 37° C; thereafter, all dilutions showing bacterial growth were inoculated onto MacConkey agar (Oxoid). Identification and typing were performed by means of standard bacteriological techniques[8]. All results were expressed as the $\log_{10}$ of the number of organisms per gram (wet weight) of faeces. In this study, we defined a 'resident' organism as an identical typed bacterium which remained in the intestinal tract for more than 4 weeks. *Enterobacteriaceae* types appearing only sporadically in the duration of the 6 weeks study were defined as 'transient' ones[9].

Studies on serum antibodies (to endogenous Enterobacteriaceae)

1. Sera

Sera were obtained once during the study from the 30 subjects. Sera were heated at 56° C for 30 minutes to inactivate complement and were stored in small aliquots at —20° C.

2. Enterobacterial antigens

All *Enterobacteriaceae* strains (residents as well as transients recovered from the 30 subjects own faeces were used. Suspensions of 10^6 bacteria per ml of demineralized water were prepared. Fat-free slides were covered with 0.05 ml of suspension. The antigen-slides were dried, treated with 96% of ethanol (fixing and killing of bacteria) and thrice washed.

3. Fluorescent anti-immunoglobulin sera

Fluoresceine-conjugated pig antisera specific for human IgM, IgG, IgA and Ig total (IgT) were supplied by Dako Laboratories (Copenhagen, Denmark). These antisera were stored at —20° C, in small quantities, which were thawed and diluted in PBS (IgM 1:80; IgG 1:100; IgA and IgT 1:120) just before each test.

4. Immunofluorescent staining procedure

The slides with enterobacterial antigens were covered with the corresponding sera obtained from the same individual from whom the *Enterobacteriaceae* spp had been isolated (serially diluted, 1:2), for 45 minutes at room temperature and thrice washed in 0.01 M phosphate buffered saline (PBS) solution (ph 8.5) for 10 minutes. Fluorescent antiglobulin was then applied for 1 hour at room temperature. The slides were washed and dried as before. A drop of mounting fluid (1 part of glycerol to 9 parts of carbonate buffer, ph 9) was placed over the area and a cover slip was added. The slides were then stored at 4° C until they were examined by immunofluorescence (IF). Titres were read as the reciprocal of the highest dilution showing fluorescence that scored 2+ or greater (on a scale of 1+ to 4+). Negative control slides that consisted of bacteria incubated first with PBS and then with the fluorescent reagents were included in each test.

Statistics

For comparison of the groups the Wilcoxon two sample test was applied to maximal titre values found within each subject.

RESULT

Bacteriological findings

Longitudinal (minimum 6 weeks) qualitative and quantitative culturing on 324 samples (10.8 samples per individual) was carried out. Figure 1 shows *Enterobacteriaceae* colonization patterns in normal subjects (a), in Crohn's disease patients (b) and in ulcerative colitis patients (c). The flora of the IBD patients was similar to that of healthy subjects. The resident flora in both groups included *Escherichia coli* only. The two most common resident *E. coli* strains (API-profiles 5144572 and 5044552) were identical in both groups. Sixteen resident endogenous *E. coli* were isolated from the IBD- and control group, i.e., on an average, 1.4 resident *E. coli* per individual. The transient flora however, consisted not only of *E. coli* but also of *Klebsiella* spp, *Enterobacter* spp, *Citrobacter* spp etc. One hundred sixty one different transient bacteria were isolated from the 30 subjects (an average of 5.3 strain per individual).

Serological findings

Sera from the 20 patients were assayed for the presence of circulating antibodies against all endogenous (resident/transient) *Enterobacteriaceae* and compared with sera from the 10 control subjects. The titres of circulating antibodies (immunoglobulin classes IgM, IgG and IgA) to all endogenous *Enterobacteriaceae* strains are shown in Figure 2. IgM and IgG antibodies predominated quantitatively over IgA antibodies. Antibody titres (IgG and IgA) to resident endogenous bacteria were significantly higher in the patient groups than in the healthy controls ($P < 0.025$, one-sided). Differences were not significant between the two groups in the IgM (and IgT) component ($P > 0.10$). Between ulcerative colitis - and Crohn's disease patients no difference was found ($P > 0.10$). Antibody titres to transient endogenous microorganisms did not differ ($P > 0.10$ in all classes of the immunoglobulins). These results indicate that both the ulcerative colitis patients and the Crohn's disease group are immunologically more responsive to endogenous resident *Enterobacteriaceae* bacteria (or antigens) compared with controls.

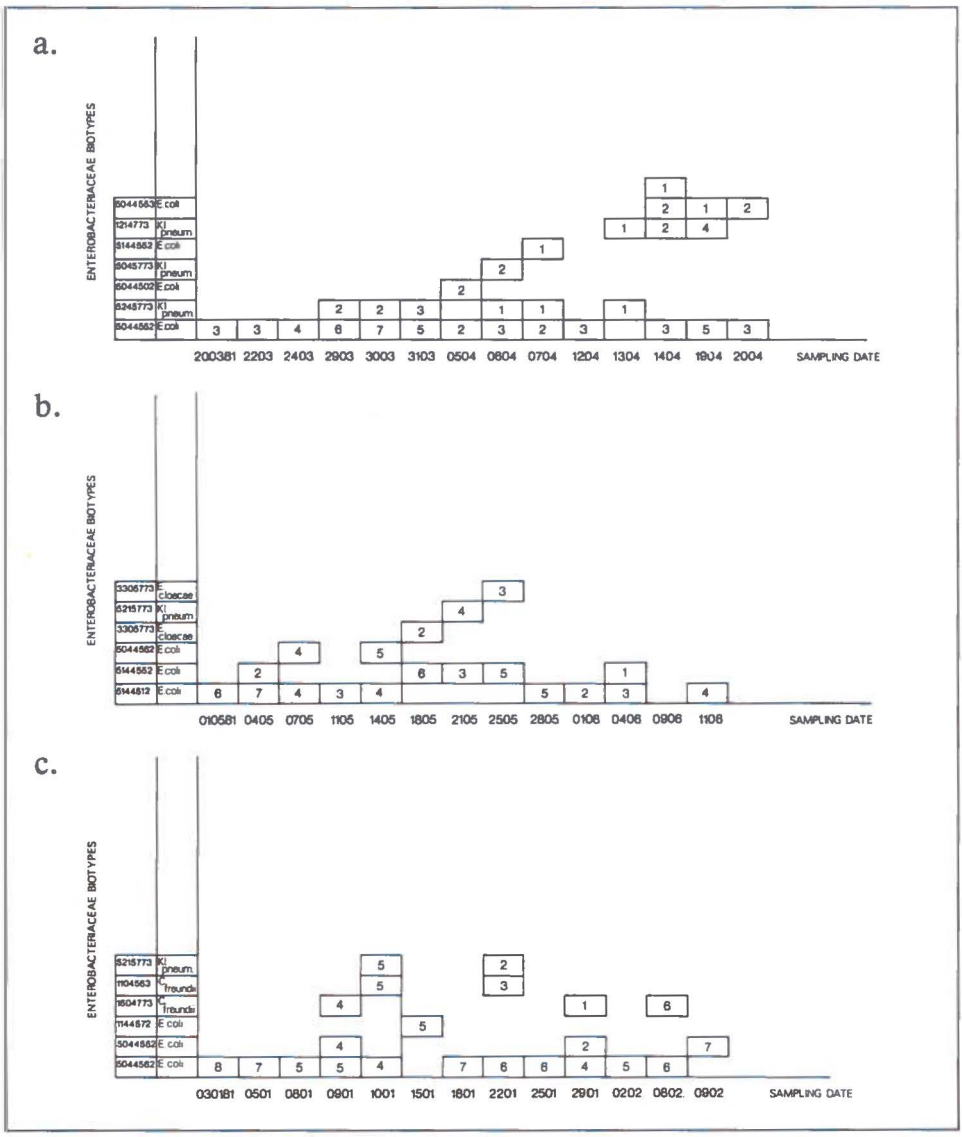


FIGURE 1

Digestive tract colonization by *Enterobacteriaceae.* Numbers in blocks give the concentration of *Enterobacteriaceae* expressed as the $\log_{10}$ of bacteria per gram of faeces.

Colonization pattern of digestive tract of a healthy subject (a), of a Crohn's disease patient (b) and of a colitis ulcerosa patient (c). Each individual is colonized by one resident *Escherichia coli* strain and by an average of four transient *Enterobacteriaceae.*

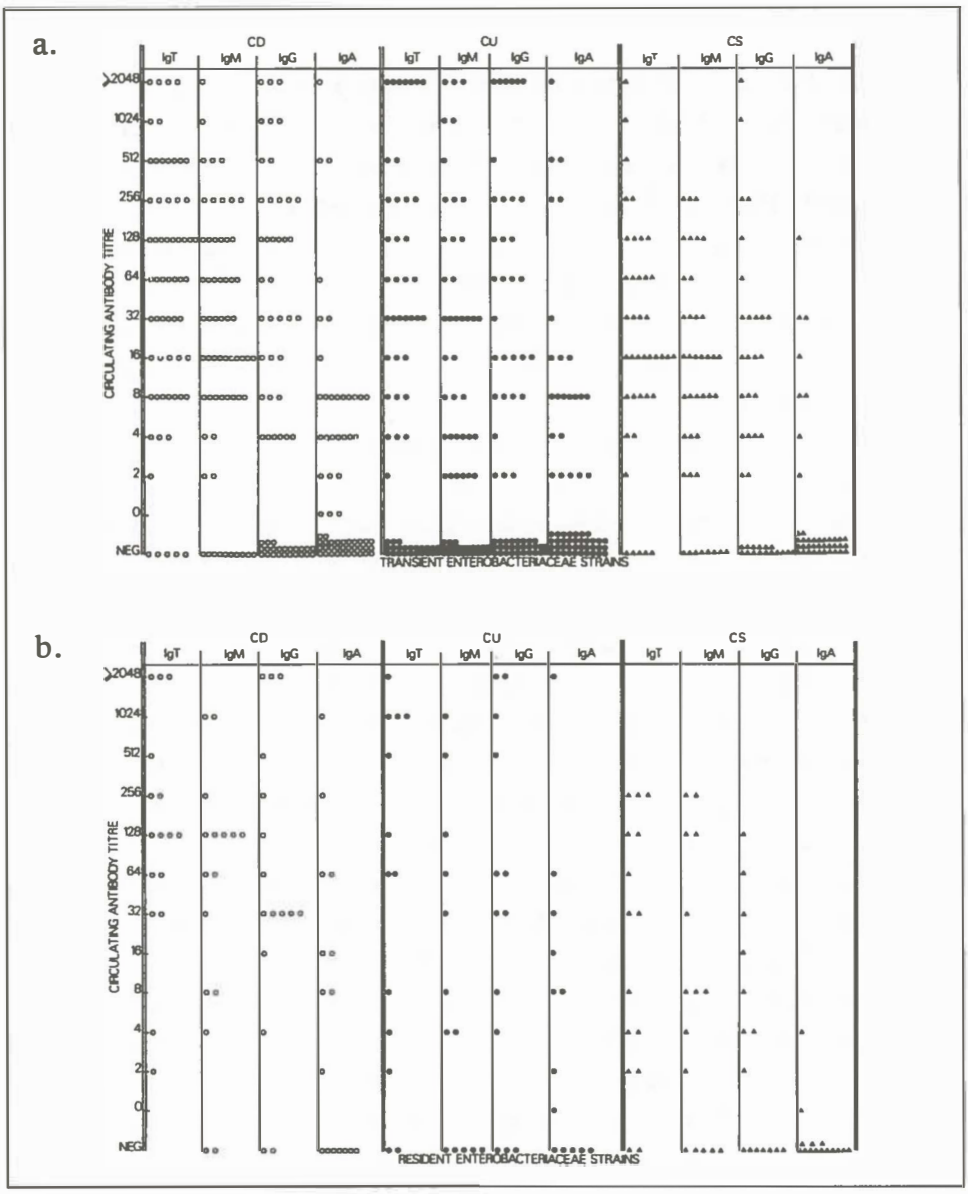


FIGURE 2

(a) circulating antibodies to resident endogenous *Enterobacteriaceae* strains.
(b) circulating antibodies to transient endogenous *Enterobacteriaceae* strains.
CD: Crohn's disease: ○
CU: colitis ulcerosa: ●
CS: control subjects: Δ

DISCUSSION

Circulating antibodies to *Enterobacteriaceae* (or enterobacterial antigens) have been demonstrated in the serum of normal subjects[10,11,12,13] and of patients with inflammatory bowel disease[14,15,16,17,18]. In all these studies only one *Enterobacteriaceae* species, namely *E. coli* was investigated. Practically all internationally accepted *E. coli* serotypes (159 supplied from laboratory stock) have been tested. Also to the different *E. coli* antigens circulating antibodies have been demonstrated: O,K,H and enterobacterial common antigen have been used as antigens.

In our investigation, sera were assayed for the presence of circulating antibodies not against laboratory strains but to the individuals endogenous *Enterobacteriaceae* strains. The type of enterobacterial antigen to which antibodies were demonstrated in this study has probably been enterobacterial surface antigen (O-antigens). That we did not investigate specific enterobacterial components but that we focused on the immunologic responsiveness of the host to his own endogenous strains, is noteworthy. Serological techniques such as indirect immunofluorescent assay (IFA) demonstrate serotypes providing O-antigens. In the present study, the API method based on biochemical reactions was used. There were two reasons why the biotyping system was preferred: its technical practicability and its relatively low costs. Moreover, the literature data indicate that biotypes often run parallel to serotypes[19,20].

The findings of this study indicate that Crohn's disease patients and ulcerative colitis patients are immunologically more responsive to antigens of endogenous *E. coli* strains than was found in the healthy control group. The significantly increased titres of circulating antibodies of IgG and IgA class to colonizing *Enterobacteriaceae* spp found in our IBD patients imply a response of the central immune apparatus to T-cell dependent antigens associated with these bacterial cell walls. These titres for most part may have been due to a secondary response which could also explain the relative low IgM titres[21]. Significantly increased circulating antibodies against endogenous (resident) *E. coli* strains can only arise following (i) increased

translocation[22] (under normal circumstances translocation from the digestive tract into the lymphoid organs occurs at low scale) and/or abnormalities of immunoregulatory T-cells[23].

Earlier studies of our group have shown that the activity of gut associated lymphoid tissue is decreased (in *in vivo* coating of endogenous *E. coli* with secretory immunoglobulin A) in IBD patients[24]. Decreased s-IgA coating of *E. coli* could involve an enhanced adherence of *E. coli* to digestive tract mucosa. Enhanced adherence is always associated with increased concentrations of *Enterobacteriaceae* spp ($\geqslant 10^6$ of *E. coli* per gram of faeces). This condition — when it concerns potentially pathogenic microorganisms — is — at least in experimental animals — associated with translocation from the digestive tract into lymphoid organs[25]. Increased translocation is very likely to be followed by increased titres of circulating immunoglobulins[26].

The finding that the colonization resistance of the digestive tract of IBD patients to *Enterobacteriaceae* spp and other potentially pathogenic microorganisms appeared unaltered, implies a normal colonization pattern of the aerobic flora, i.e., no colonizing *E. coli* in the oropharynx and intestinal *E. coli* concentrations of $< 10^6$ per gram of faeces. In other words, *Enterobacteriaceae* exposition in IBD patients did not differ from that in healthy subjects. Increased translocation of *Enterobacteriaceae* as a reason to explain the high IgG and IgA serum antibodies seems to be very improbable.

It seems more likely that our findings are due to a disorder of the immunoregulatory T-cells[27]. A decrease of T-suppressor cell activity as described by several authors[28,29] may explain the significantly increased IgG and IgA titres to endogenous (colonizing) *Enterobacteriaceae* spp found in our patients. The decreased secretion of s-IgA into the digestive tract resulting into a decreased s-IgA coated intestinal bacteria, described elsewhere, could be due to either a decreased IgA secretion (as a consequence of a decreased number of T-helper cells)[30] or a decreased coupling of IgA to secretory component[31,32].

There is evidence suggesting that T-suppressor cells are not only involved in normal B-cell activity but also in tolerance[33,34]. In this regard T-suppressor cells may control the auto-antibody precursor

B-cells from becoming active. This makes likely that the spontaneous onset of any auto-immune disease is related to a decrease or loss of suppressor cell function[35]. It seems probable that inflammatory bowel disease has here a common denominator with auto-immunity. Both may result from a similar diathesis[36]. Whether this is genetically determined or this is due to a viral infection (*Herpes* virus) of the lymphoid system is unknown. Perhaps both are involved.

The decreased GALT function in association with the indirect evidence for a decrease of T-suppressor cell activity make likely that in IBD a T-cell disorder may play a role. The T-cell response to an intestinal antigen has two important functions[36]. T-helper cells aid in the formation of IgA antibody production which acts locally to reduce adherence, colonization and subsequent translocation from the intestines. T-suppressor cells act by suppressing the formation of other classes (IgG, IgM) of circulating antibody. This supports the function of locally excreted s-IgA and further ensures that the small quantities of antigen which are translocated across the mucosa do not provoke possibly damaging inflammation responses[37,38]. A defect in that way that T-cells perform these tasks may underlie the altered immune responsiveness characteristic of inflammatory bowel disease.

In conclusion: this investigation indicates that the aerobic *Enterobacteriaceae* spp are probably not pathogenetically involved in IBD. However, they are good indices of altered immunologic responsiveness of the host to his own digestive tract flora.

ACKNOWLEDGEMENTS

We thank the nursing staff and the volunteers for their collaboration and we want to express our appreciation to Mrs. E. van Santen, Mrs. M. Wiersma and Mr. J. Krikken for technical assistance.

REFERENCES

[1] SHORTER, R.G., HUIZENGA, K.A., SPENCER, R.J. (1972). A working hypothesis for the etiology and pathogenesis of nonspecific inflammatory bowel disease. *Digestive diseases,* 17:1024-1032.

[2] SHORTER, R.G. (1974). Immunological aspects of gastrointestinal disease: an up-to-date account of inflammatory diseases such as ulcerative colitis and Crohn's disease. In: *Progress in Immunology* II. Eds. Brent, L., Holborow, J. North-Holland Publishing Company, Amsterdam, 4:209-219.

[3] VAN DER WAAIJ, D., TIELEMAN-SPELTIE, T.M., DE ROECK-HOUBEN, A.M.J. (1978). Relation between the faecal concentration of various potentially pathogenic microorganisms and infections in individuals (mice) with severely decreased resistance to infection. *Antonie van Leeuwenhoek,* 44:395-405.

[4] MYEROWITZ, R.L., MEDEIROS, A.A., O'BRIEN, T.F. (1971). Recent experience with bacillemia due to gram-negative organisms. *J.Inf.Dis.,* 124:239-246.

[5] VAN DER WAAIJ, D., HEIDT, P.J. (1977). Intestinal bacterial ecology in relation to immunological factors and others defence mechanisms. In: *Food and Immunology*. Eds. Hambraeus, L., Hanson, L.A. Almqvist & Wiksell, Stockholm, 133-141.

[6] VAN SAENE, H.K.F., VAN DER WAAIJ, D. (1979). A novel technique for detecting s-IgA coated potentially pathogenic microorganisms in the human intestine. *J.Immunol.Methods,* 30:87-96.

[7] GOOD, R.A., RODEY, G.E. (1970). IgA deficiency, antigenic barriers, and autoimmunity. *Cell.Immunol.,* 1:147-148.

[8] SMITH, P.B., TOMFOHRDE, K.M., RHODEN, D.L., BALOWS, A. (1972). API system: a multitube micromethod for identification of *Enterobacteriaceae. Appl.Microbiol.,* 24:449-552.

[9] ROBINET, H.G. (1962). Relationship of host antibody to fluctuations of *Escherichia coli* serotypes of human intestine. *J.Bacteriol.,* 84:896-901.

[10] MICHAEL, J.G., WHITBEY, J.L., LANDY, M. (1962). Studies on natural antibodies to Gram-negative bacteria. *J.Exp.Med.,* 155:131-146.

[11] COHEN, I.R., NORRINS, L.C. (1966). Natural human antibodies to Gram-negative bacteria: immunoglobulins G, A and M. *Science,* 152:1257-1259.

[12] KUNIN, G.M. (1962). Antibody distribution against non-enteropathic *E. coli. Arch.Int.Med.,* 110:676-686.

[13] BROWN, W.R., LEE, E.M. (1973). Radioimmunologic measurements of naturally occuring bacterial antibodies. I. Human serum antibodies reactive with *Escherichia coli* in gastrointestinal and immunological disorders. *J.Lab.Clin.Med.,* 82:125-136.

[14] PERLMANN, P., HAMMARSTROM, S., LAGERCRANTZ, R., GUSTAFSSON, B.E. (1965). Antigen from colon of germ-free rats and antibodies in human ulcerative colitis. *Ann.N.Y.Acad.Sci.,* 124:377-394.

[15] PERSSON, S., DANIELLSON, D. (1979). On the occurrence of serum antibodies to *Bacteroides fragilis* and serogroups of *E. coli* in patients with Crohn's disease. *Scand.J.Inf.Dis.,* 19:61-67 (Suppl.).

[16] DANIELLSON, D., KJELLANDER, J., PERSSON, S., WALLENSTEN, S. (1979). Investigation of the immune response to aerobic and anaerobic intestinal bacteria in a patient with Crohn's disease. *Scand.J.Inf.Dis.,* 19:52-60.

[17] HEDDLE, R.J., SHEARMAN, D.J.C. (1979). Serum antibodies to *Escherichia coli* in subjects with ulcerative colitis. *Clin.Exp.Immunol.,* 38:22-30.

[18] TABAQCHALI, S., O'DONOGHUE, P., BETTELHEIM, K.A. (1978). *Escherichia coli* antibodies in patients with inflammatory bowel disease. *Gut,* 19:108-113.

[19] BETTELHEIM, K.A., TAYLOR, J. (1969). A study of *Escherichia coli* isolated from chronic urinary infection. *J.Med.Microbiol.,* 2:225-236.

[20] VAN DER WAAIJ, D., SPELTIE, T.M., GUINEE, P.A.M., AGTERBERG, C. (1975). Serotyping and biotyping of 160 *Escherichia coli* strains: comparative study. *J.Clin.Microbiol.,* 1:237-238.

[21] GELL, P.G.H., COOMBS, R.R.A., LACHMANN, P.J. (1975). *Clinical aspects of immunology.* Blackwell, Oxford.

[22] BERG, R.D., GARLINGTON, A.W. (1979). Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. *Infect.Immun.,* 23:403-411.

[23] WALDMANN, T.A., BRODER, S. (1977). Suppressor cells in the regulation of the immune response. *Prog.Clin.Immunol.,* 3:155-199.

[24] VAN SAENE, H.K.F., JANSEN, W., FIDLER, V., VEEGER, W.C., VAN DER WAAIJ, D. (1982). Evidence for dysfunction of gut associated lymphoid tissue in inflammatory bowel disease patients. Submitted.

[25] OWENS, W.E., BERG, R.D. (1980). Bacterial translocation from the gastrointestinal tract of athymic (nu/nu) mice. *Infect.Immun.,* 27:461-467.

[26] BERG, R.D. (1979). Ph.D. thesis.

[27] REINHERZ, E.L., SCHLOSSMAN, S.F. (1980). Current concepts in immunology. Regulation of the immune response: inducer and suppressor T-lymphocyte subsets in human beings. *New Engl.J.Med.,* 303:370-372.

[28] HODGSON, H.J.F., WANDS, J.R., ISSELBACHER, K.J. (1978). Decreased suppressor cell activity in inflammatory bowel disease. *Clin.Exp.Immunol.,* 32:451-458.

[29] VICTORINO, R.M.M., HODGSON, H.J.F. (1981). Spontaneous suppressor cell function in inflammatory bowel disease. *Dig.Dis.Sci.,* 26:801-806.

[30] ELSON, C.O., HECK, J.A., STROBER, W. (1979). T-cell regulation of murine IgA synthesis. *J.Exp.Med.,* 149:632-643.

[31] ENGSTROM, J.F., ARVANITAKIS, C., SAGAWA, A., ABDOU, N.I. (1978). Secretory immunoglobulin deficiency in a family with inflammatory bowel disease. *Gastroenterology,* 74:747-751.

[32] HEREMANS, J.F. (1975). The secretory immune system. A critical appraisal. In: *The immune system and infectious diseases.* Karger, Basel, 376-385.

[33] ASHERSON, G., ZEMBALA, L.M., PERERA, M.A.C.C., MAYHEW, B., THOMAS, W.R. (1977). Production of immunity and unresponsiveness in the mouse by feeding contact sensitizing agents and the role of suppressor cells in the Peyer's patches, mesenteric lymph nodes and other lymphoid tissues. *Cell.Immunol.,* 33:145.

[34] Immunological tolerance. (1976). *British Medical Bulletin,* 32.

[35] RAEMAN, F., DE COCK, W., DE BEUKELAAR, T., DE CREE, J., VERHAEGEN, H. (1981). Enumeration of T-lymphocytes and T-lymphocyte subsets in auto-immunity disease using monoclonal antibodies. *Clin.Exp.Immunol.,* 45:475-479.

[36] LANCET (1979). Editorial. Gut reaction to antigen. *Lancet,* i:763.

[37] SWARBRICK, E.T., STOKES, C.R., SOOTHILL, J.F. (1979). Absorption of antigens after oral immunisation and the simultaneous induction of specific systemic tolerance. *Gut,* 20:121-125.

[38] CHALLACOMBE, S.J., TOMASI, T.B. (1980). Systemic tolerance and secretory immunity after oral immunization. *J.Exp.Med.,* 152:1459-1472.

CHAPTER SIX

GENERAL DISCUSSION

,,even if a specific agent could be found ininflammatory bowel diseases, host immune responses would be likely to be involved''

Shorter, R.G.

The aim of this investigation was to study which part of the colonising (indigenous) flora (aerobes and anaerobes) can play a role in the aetiology and pathogenesis of inflammatory bowel diseases. This investigation was merely based on the working hypothesis of Shorter[1,2]. Inflammatory bowel diseases could be regarded as expression forms of immune reactivity ('hypersensitivity reactions') to endogenous intestinal bacterial antigens. In (genetically?) predisposed individuals with a decreased function of gut associated lymphoid tissue (GALT), translocation of bacterial antigens may occur and result in circulating antibodies and according to Shorter into stimulation of T-cells. Endogenous *Enterobacteriaceae* spp sharing antigens with host constituents thus may induce cross-reacting antibodies, i.e., antibodies which also complex with host (organ or tissue specific) antigens. These cross-reacting antibodies could explain the immunological inflammation reactions (granuloma, ulcer) seen in IBD[3,4].

In this investigation we focused our research on three aspects possibly involved in the pathogenesis:

(i) *antigen*. An inventory of the aerobic digestive tract flora is made. The colonization pattern of endogenous *Enterobacteriaceae* spp was determined, in order to evaluate the enterobacterial antigen load

inside the lumen of the digestive tract.

(ii) *local immune system*. The function of the local immune system was measured by investigating the percentage of secretory immunoglobulin A (s-IgA) coated *Enterobacteriaceae* spp.

(iii) *central immune system*. The immunological responsiveness to endogenous *Enterobacteriaceae* spp was measured by determining titres of circulating antibodies to endogenous *Enterobacteriaceae* spp.

The working hypothesis of this investigation was that aerobic *Enterobacteriaceae* spp may increasingly translocate in IBD patients. A hypersensitivity reaction to these bacterial antigens in the gut wall could result from the increased translocation of intestinal bacteria into lymphoid organs, i.e., passage of bacteria from the digestive tract lumen through the epithelium into the systemic immune system (e.g., mesenteric lymph nodes, spleen) inducing an antibody response[5,6,7,8].

Three observations made in this study, however, make a central role of Gram-negative *Enterobacteriaceae* spp in the aetiology and pathogenesis of IBDs unlikely. (i) The aerobic flora was found qualitatively normal (Chapter three). (ii) The local immune system however appeared deficient both when the disease was in relapse as well as in remission (Chapter four). (iii) IBD patients were found immunologically more responsive to endogenous *Enterobacteriaceae* spp than healthy subjects (Chapter five).

ad i. Our findings make an increased translocation of *Enterobacteriaceae* spp unlikely. For, the most important condition necessary for enhanced translocation is the presence of high numbers of adhering bacteria reflected by the high concentrations of bacteria within the digestive tract lumen (faeces)[9,10]. In all 66 IBD patients studied the aerobic *Enterobacteriaceae* spp concentrations in the faeces were completely normal in comparison with healthy controls. These observations confirm earlier studies[11,12,13].

ad ii. The findings in the immunological part of this study — decreased GALT and increased circulating antibody titres — suggest that IBDs are associated with a T-cell disorder. IgA appears to be T-cell dependent[14,15]. A deficiency of local gut associated immunity has earlier also been described by others[16,17,18,19,20]. Our findings of decreased s-IgA coated intestinal Gram-negative bacteria fit very well with these earlier

observations. The low concentrations of secretory component in saliva however, may indicate that this low coating incidence could also be due to decreased secretion of secretory component.

ad iii. The observation of increased antibody titres of IgG and IgA may imply a decreased number of (IgG and IgA) T-suppressor cells[21,22]. In this respect there could be a relation with auto-immune diseases in which also strong evidence exists of a decrease of T-suppressor cell activity[23,24,25]. Recently, several authors have also reported a dysregulation of the delicate balance of immune homeostasis between T-helper and T-suppressor in IBD patients[26,27,28,29,30]. A decrease of T-suppressor cell activity in combination with a dysfunction of local gut immunity may be of key importance in the pathogenesis.

The two immunological aberrations — found in this study by means of aerobic *Enterobacteriaceae* spp — make unlikely that the aerobic *Enterobacteriaceae* spp are involved in the pathogenesis. This conclusion is based on the completely normal colonization pattern of the digestive tract in IBD patients. If bacteria are involved in the pathogenesis, the numerically most prominent anaerobes are the likely candidates. This part of the digestive tract microflora consists of many mucosa adhering species, forming the 'wall paper' of the digestive tract. Consequently, the anaerobic part of the flora is characterised by constantly high concentrations ($\geqslant 10^{10}$ per gram of faeces)[31,32,33]. In contrast to *Enterobacteriaceae* spp, anaerobic bacteria in faeces of healthy subjects are not coated with s-IgA[34,35]. Since anaerobic bacteria massively adhere to the mucosa and may pass the M cell lining which covers the Peyer's patches to get into the patch itself, they are likely to induce s-IgA production, however they do not. This suggests that under normal circumstances a form of immunological tolerance of the host organism against his adhering anaerobes exists. Tolerance could be due to (early) development of specific T-suppressor cells. The latter would suppress both local as well as systemic immune responses[36,37].

The results of our investigation provide — in an indirect way — supportive evidence for the existence of a decreased T-suppressor cell activity; i.e., increased serum titres of IgG and IgA against *Enterobacteriaceae* spp (Chapter five). Increased circulating antibodies have also

been reported by several others[38,39,40].
A continuous translocation of one or more anaerobic bacterial strains or absorption of their cell wall components from the digestive tract into submucosa and lymphoid organs (mesenteric lymph nodes and spleen). This could result in complement fixing antibody formation which could explain the development of a state of hypersensitivity against (anaerobic) intestinal bacterial antigens[41,42,43]. As soon as complement fixing antibodies to these anaerobes are being produced and circulate, they may complex with the continuing anaerobic (antigenic) penetration or absorption. Observations by Wensinck and van de Merwe make likely that the anaerobes are indeed propable candidates for translocation and induction of antibodies in IBD patients[44,45,46,47]. Their conclusion regarding the possible role of anaerobes in the pathogenesis of IBDs complements the observations made in this investigation.

In conclusion: it is not likely that the *Enterobacteriaceae* spp are directly involved in the pathogenesis of inflammatory bowel diseases. However, they have served as good indices for the monitoring of the altered immunological responsiveness of the IBD patient. This study confirms the conclusion of others that a T-cell disorder may play a central role in the pathogenesis of IBD[48,49,50,51,52].

From our bacteriological point of view it seems conceivable that either the restoration of the immunne disorder(s) or the elimination of the anaerobic species to which the host could be hypersensitive from the digestive tract may cause longterm remission. Restoration of secretory component secretion is for the time being not possible. However, attempts could be made to eliminate the anaerobic species against which the patient may have titres of circulating antibodies. Selective elimination of one or more anaerobic bacterial species (involved in the pathogenesis) is also impossible. A more radical approach, total decontamination of the digestive tract, is worth consideration[51]. By means of broad spectrum non-absorbable antimicrobial drugs the aerobic and anaerobic flora can be eliminated from the intestinal tract[52]. Total decontamination however requires strict isolation of the patient to pre-

vent overgrowth by resistant strains. Once the lesions are healed the patient could be recolonised stepwise with (colonization resistance supporting) intestinal 'wall paper' anaerobes. Those strains however, to which the IBD patient has circulating (complement fixing) antibodies so that he may respond with a hypersensitive inflammatory reaction, should be omitted[53]. This condition should be continued until the specific antibodies have disappeared from the circulation.

REFERENCES

[1] SHORTER, R.G., HUIZENGA, K.A., SPENCER, R.J. (1972). A working hypothesis for the etiology and pathogenesis of nonspecific inflammatory bowel disease. *Digestive Diseases,* 17:1024-1032.

[2] SHORTER, R.G. (1974). Immunological aspects of gastrointestinal disease: an up-to-date account of inflammatory diseases such as ulcerative colitis and Crohn's disease. In: *Progress in Immunology* II. Eds. Brent, L., Holborow, J. North-Holland Publishing Company, Amsterdam, 4:209-219.

[3] KIRSNER, J.B., SHORTER, R.G. (1982). Recent developments in nonspecific inflammatory bowel disease. *New Engl.J.Med.,* 306:837-848.

[4] SCHACHTER, H., KIRSNER, J.B. (1980). *Crohn's disease of the gastrointestinal tract.* Wiley & Sons, New York.

[5] BERG, R.D., GARLINGTON, A.W. (1979). Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. *Infect.Immun.,* 23:403-411.

[6] VAN DER WAAIJ, D., BERGHUIS, J.M., LEKKERKERK, J.E.C. (1972). Colonization resistance of the digestive tract and the occurrence of spread of bacteria to lymphatic organs in mice. *J.Hyg.,* 70:55-60.

[7] OWENS, W.E., BERG, R.D. (1980). Bacterial translocation from the gastrointestinal tract of athymic (nu/nu) mice. *Infect.Immun.,* 27:461-467.

[8] BERG, R.D. (1979). Ph.D. thesis.

[9] VAN DER WAAIJ, D., TIELEMAN-SPELTIE, T.M., DE ROECK-HOUBEN, A.M.J. (1978). Relation between the faecal concentration of various potentially pathogenic microorganisms and infections in individuals (mice) with severely decreased resistance to infection. *Antonie van Leeuwenhoek,* 44:395-405.

[10] BERG, R.D., GARLINGTON, A.W. (1979). Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic or antibiotic decontaminated mice. *Infect.Immun.,* 29:1073-1081.

[11] COOKE, E.M. (1967). A quantitative comparison of the faecal flora of patients with ulcerative colitis and that of normal persons. *J.Path.Bact.,* 94:439-444.

[12] GORBACH, S.L., NAHAS, L., PLAUT, A.G., WEINSTEIN, L., PATTERSON, J.F., LEVITAN, R. (1968). Studies on the intestinal microflora: V. Fecal microbial ecology in ulcerative colitis and regional enteritis: relationship to severity of disease and chemotherapy. *Gastroenterology,* 54:575-587.

[13] VINCE, A., DYER, N.H., O'GRADY, F.W., DAWSON, A.M. (1972). Bacteriological studies in Crohn's disease. *J.Med.Microbiol.,* 5:219-229.

[14] PRITCHARD, H., RIDDAWAY, J., MICKLEM, H.S. (1973). Immune responses in congenitally thymus-less mice. II. Quantitative studies of serum immunoglobulins, the antibody response to sheep erytrocytes, and the effect of thymus allografting. *Clin.Exp.Immunol.,* 13:125-138.

[15] GUY-GRAND, D., GRISCELLI, C., VASSALLI, P. (1975). Peyer's patches, gut IgA plasma cells and thymic function: study in mice bearing thymic grafts. *J.Immunol.,* 115:361-364.

[16] GREEN, F.H.Y., FOX, H. (1975). The distribution of mucosal antibodies in the bowel of patients with Crohn's disease. *Gut,* 16:125-131.

[17] GELZAYD, E.A., KRAFT, S.C., FITCH, F.W., KIRSNER, J.B. (1968). Distribution of immunoglobulins in human rectal mucosa. II. Ulcerative colitis and abnormal mucosal control subjects. *Gastroenterology,* 54:341-347.

[18] DAS, K.M., ERBER, W.F., RUBINSTEIN, A. (1977). Immunohistochemical changes in morphologically involved and uninvolved colonic mucosa of patients with idiopathic proctitis. *J.Clin.Invest.,* 59:379-385.

[19] ENGSTROM, J.F., ARVANITAKIS, C., SAGAWA, A., ABDOU, N.I. (1978). Secretory immunoglobulin deficiency in a family with inflammatory bowel disease. *Gastroenterology,* 74:747-751.

[20] MacDERMOTT, R.P., NASH, G.S., BERTOVICH, M.J., SEIDEN, M.V., BRAGDON, M.J., BEALE, M.G. (1981). Alterations of IgM, IgG, and IgA synthesis and secretion by peripheral blood and intestinal mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Gastroenterology,* 81:844-852.

[21] WALDMANN, T.A., BRODER, S. (1977). Suppressor cells in the regulation of the immune response. *Prog.Clin.Immunol.,* 3:155-199.

[22] REINHERZ, E.L., SCHLOSSMAN, S.F. (1980). Current concepts in immunology. Regulation of the immune response: inducer and suppressor T-lymphocyte subsets in human beings. *New Engl.J.Med.,* 303:370-372.

[23] RAEMAN, F., DE COCK, W., DE BEUKELAAR, T., DE CREE, J., VERHAEGEN, H. (1981). Enumeration of T-lymphocytes and T-lymphocyte subsets in auto-immunity disease using monoclonal antibodies. *Clin.Exp.Immunol.,* 45:475-479.

[24] BRESNIHAN, B., JASIN, H.E. (1977). Suppressor function of peripheral blood mononuclear cells in normal individuals and patients with systemic lupus erythematosus. *J.Clin.Invest.,* 59:106-116.

[25] REINHERZ, E.L., PARKMAN, R., RAPPEPORT, J., ROSEN, F.S., SCHLOSSMAN, S.F. (1979). Aberrations of suppressor T-cells in human graft-versus-host disease. *New Engl.J.Med.,* 300:1061-1068.

[26] HODGSON, H.J.F., WANDS, J.R., ISSELBACHER, K.J. (1978). Decreased suppressor cell activity in inflammatory bowel disease. *Clin.Exp.Immunol.,* 32:451-458.

[27] VICTORINO, R.M.M., HODGSON, H.J.F. (1981). Spontaneous suppressor cell function in inflammatory bowel disease. *Dig.Dis.Sci.,* 26:801-806.

[28] GOODACRE, R., BIENENSTOCK, J. (1978). Loss of suppressor cell activity in intestinal lymphocytes from patients with Crohn's disease. *Gut,* 20:A910, abstract.

[29] SHORTER, R.G., REMINE, S.G., BARTNIK, W. (1981). T-cells and concanavalin A - induced suppressor cell activity in vitro in colonic inflammatory bowel disease and in colorectal carcinoma. In: *Recent advances in Crohn's disease.* Eds. Pena, A.S., Weterman, I.T., Booth, C.C., Strober, W. Martinus Nijhoff, The Hague, 448-455.

[30] PENA, A.S., GNOSSEN, J., DAMSTEEG, W.G.M., WETERMAN, I.T., MEIJER, C.L.M. (1981). T-cell subpopulations in Crohn's disease. In: *Recent advances in Crohn's disease.* Eds. Pena, A.S., Weterman, I.T., Booth, C.C., Strober, W. Martinus Nijhoff, The Hague, 403-406.

[31] GALL, L.S. (1970). Normal fecal flora of man. *Am.J.Clin.Nutr.,* 23:1457-1465.

[32] MOORE, W.E.C., HOLDEMAN, L.V. (1974). Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl.Microbiol.,* 27:961-979.

[33] GORBACH, S.L. (1969). On the intestinal flora. *Gastroenterology,* 57:231-232.

[34] VAN SAENE, H.K.F., VAN DER WAAIJ, D. (1982). *In vivo* binding of secretory immunoglobulin A to normal gastrointestinal flora. *Gut,* in press.

[35] VAN DER WAAIJ, D., HEIDT, P.J. (1977). Intestinal bacterial ecology in relation to immunological factors and other defense mechanisms. In: *Food and Immunology.* Eds. Hambraeus, L., Hanson, L.A. Almqvist & Wiksell, Stockholm, 133-141.

[36] OPSTELTEN, D. (1981). Germinal centers and B-lymphocyte differentiation in the rabbit. Ph. D. thesis, Groningen, The Netherlands.

[37] GORZINSKY, E.A., NETER, E., AMBRUS, J.L. (1970). Differences in antibody responses of mouse strains to enterobacterial common antigen. *Proc.Soc.Exp.Biol. Med.,* 134:776-779.

[38] HEDDLE, R.J., SHEARMAN, D.J.C. (1979). Serum antibodies to *Escherichia coli* in subjects with ulcerative colitis. *Clin.Exp.Immunol.,* 38:22-30.

[39] TABAQCHALI, S., O'DONOGHUE, P., BETTELHEIM, K.A. (1978). *Escherichia coli* antibodies in patients with inflammatory bowel disease. *Gut,* 19:108-113.

[40] DANIELSSON, D., KJELLANDER, J., PERSSON, S., WALLENSTEN, S. (1979). Investigation of the immune response to aerobic and anaerobic intestinal bacteria in a patient with Crohn's disease. *Scand.J.Infect.Dis.,* 19(S):52-60.

[41] WENSINCK, F. (1976). Faecal flora of Crohn's patients. Serological differentiation between Crohn's disease and ulcerative colitis. In: *The management of Crohn's disease.* Eds. Weterman, I.T., Pena, A.S., Booth, C.C. Excerpta Medica, Amsterdam, 103-107.

[42] MATTHEWS, N., MAYBERRY, J.F., RHODES, J., NEALE, L., MUNRO, J., WENSINCK, F., LAWSON, G.H.K., ROWLAND, A.C., BERKHOFF, G.A., BARTHOLD, S.W. (1980). Agglutinins to bacteria in Crohn's disease. *Gut,* 21:376-380.

[43] HELPHINGSTINE, C.J., HENTGES, D.J., CAMPBELL, B.J., BUTT, J., BARRETT, J.T. (1979). Antibodies detectable by counterimmuno-electrophoresis against *Bacteroides* antigens in serum of patients with inflammatory bowel disease. *J.Clin.Microbiol.,* 9:373-378.

[44] WENSINCK, F. (1975). The faecal flora of patients with Crohn's disease. *Antonie van Leeuwenhoek,* 41:214-215.

[45] VAN DE MERWE, J.P. (1980). Serum antibodies to anaerobic coccoid rods in Crohn's disease. Ph.D. thesis, Rotterdam, The Netherlands.

[46] VAN DE MERWE, J.P. (1980). A possible role of *Eubacterium* and *Peptostreptococcus* spp in the aetiology of Crohn's disease. *Antonie van Leeuwenhoek,* 46:587-593.

[47] KROOK, A. (1980). Microbiological and clinical studies on Crohn's disease. Ph.D. thesis, Uppsala, Sweden.

[48] KNAPP, W., SMOLEN, J.S., LANZER, G., BERGER, R., MENZEL, E.J., GRABNER, G., GANGL, A. (1981). Con A induced suppressor cell activity in IBD and other inflammatory diseases. In: *Recent advances in Crohn's disease.* Eds. Pena, A.S., Weterman, I.T., Booth, C.C., Strober, W. Martinus Nijhoff Publishers, The Hague, 380-389.

[49] THOMAS, H.C., JEWELL, D.P. (1979). *Clinical gastrointestinal immunology.* Blackwell, Oxford, 121-163.

[50] LANCET (1979). Editorial. Gut reaction to antigens. *Lancet,* i:763.

[51] ELSON, C.O., HECK, J.A., STROBER, W. (1979). T-cell regulation of murine IgA synthesis. *J.Exp.Med.,* 149:632-643.

[52] SWARBRICK, E.T., STOKES, C.R., SOOTHILL, J.F. (1979). Absorption of antigens after oral immunisation and the simultaneous induction of specific systemic tolerance. *Gut,* 20:121-125.

[53] BODEY, G.P., GEHAN, E.A., FREIREICH, E.J., FREI, E. (1971). Protected environment-prophylactic antibiotic program in the chemotherapy of acute leukemia. *Am.J.Med.Sci.,* 262:138-151.

[54] PREISLER, H.D., GOLDSTEIN, I.M., HENDERSON, E.S. (1970). Gastrointestinal sterilization in the treatment of patients with acute leukemia. *Cancer,* 26:1076-1081.

[55] VAN DER WAAIJ, D., VOSSEN, J.M., KORTHALS ALTES, C., HARTGRINK, C. (1977). Reconventionalization following antibiotic decontamination in man and animals. *Am.J.Clin.Nutr.,* 30:1887-1895.

CHAPTER SEVEN

CONCLUDING REMARKS

„the combination of a decreased gut associated lymphoid tissue with a decrease of T-suppressor cell activity may be of primary importance in the pathogenesis of inflammatory bowel diseases''

van Saene, H.K.F.

If intestinal bacteria (or intestinal bacterial antigens) play a role in the aetiology and pathogenesis of inflammatory bowel diseases, the most likely candidates are those species which live in great numbers in intimate relation with the mucosa, i.e., the anaerobes. It is in an indirect way that we came to this conclusion. Following arguments support the conclusion of this investigation.

1. Many anaerobic species are constantly present in high concentrations ($\geqslant 10^{10}$ anaerobes per gram of faeces); many species adhere to the intestinal epithelium forming the 'wall paper' of the mucosa. Aerobic bacteria are normally found in much lower concentrations in the digestive tract ($< 10^{6}$ aerobes per gram of faeces) both in healthy subjects and in IBD patients; aerobes, when they colonise, may form only small patches in the far most part anaerobic 'wall paper'.

2. There is evidence for an immunologic tolerance for anaerobes. This may be caused by T-suppressor cells in the GALT. The function of this tolerance could be prevention of the production of IgA. IgA to anaerobes may limit their adherence and there with their protective

function. In addition they may ensue prevention of development of inflammatory (tissue damaging) reactions to otherweise beneficial (CR-constituting) bacteria or absorbed components of them. A T-helper cell response to an (aerobic bacterial) antigen has two important functions. It aids the formation of s-IgA which after excretion into the gut mucus acts locally to impair with adherence, colonization and subsequent translocation of antigen from the digestive tract. Suppression of the formation of other classes of circulating antibody may prevent that the small quantities of aerobic bacterial antigen which do translocate every now and then across the mucosa lining provoke unnecessary (damaging) inflammatory responses.

3. This investigation provides, albeit indirectly, evidence for the existence of a decrease of T-suppressor cell activity in IBD patients. I.e., the tolerance establishing an important barrier against an immune response to bacteria forming the 'wall paper' of the gut mucosa is broken at least to some anaerobic species *(Eubacterium* spp and *Peptostreptococcus* spp) in this type of patient. This break of systemic tolerance may be of key importance in the aetiology and pathogenesis of the inflammatory reactions seen in IBD.

4. High numbers of adhering anaerobes are perhaps also normally associated with translocation of these anaerobes or their antigenic components. In IBD patients anti-anaerobic antibodies may complex in the submucosa with corresponding antigens of anaerobic bacteria and ensue either a focal inflammatory reaction resulting in an ulcer or immune complexes which may deposit over larger areas to cause granuloma formation.

5. The decreased excretion of functional s-IgA in IBD patients is perhaps another factor of great importance in the pathogenesis. If, in absence of tolerance to certain adhering anaerobic bacteria, those species would induce in IBD patients secretion of specific s-IgA, the latter may interfere with their 'wall paper' function so those species would come off the wall. This would virtually eliminate the chance that antigenic components of these bacteria would get into the sub-

mucosa in sufficient amounts to cause clinical disease. At least some of these open places on the mucosa would rapidly be replaced by aerobic potentially pathogenic microorganisms. This is apparently not the case, since the numbers and concentrations of aerobic potentially pathogenic microorganisms were normal in our patient material. Consequently, not only a decreased T-suppressor cell function but also a decreased excretion of functional s-IgA may explain why the anaerobic 'wall paper' fraction to which antibodies may circulate in high titres do not come off the wall so that antigenic components are continuously formed in close vicinity to the mucosa. These anaerobes or their antigenic (cell wall) constituents may continuously translocate and cause inflammation and/or granuloma formation.

SUMMARY

This investigation concerns some facets of the aetiology of Crohn's disease and ulcerative colitis, two chronic inflammatory diseases of the alimentary canal. The working hypothesis assumes that both of these conditions are caused by immune allergic reactions to antigens normally present in the alimentary canal. These antigens, possibly of microbial (bacterial or viral) or dietary nature, could produce antibodies which react with the patient's own tissues, in this case the alimentary tract. Also because food probably is of an inconstant composition and that of the colonising bacteria is definably more consistant, we concentrated on the bacterial antigens to the endogenous flora. This antigen source is under normal circumstances held under control by the host defence mechanisms (both local and systemic). The local defence mechanism tries to keep the antigen inside the lumen of the digestive tract using secretory immunoglobulin A (s-IgA); the systemic immune mechanism (reacting to endogenous bacterial antigens with circulating antibodies) is depressed by T-suppressor cell activity. Should this immunoregulating homeostasis fail then increased translocation (i.e., passage from the lumen to the lymphatic organs) could occur. This could induce circulating antibodies and possible allergic reactions (type 2 or 3).

In this investigation we studied this interaction between gut antigens and the defence mechanisms using the *Enterobacteriaceae* species, not only as possible allergens but also as useful indices for determining (i) the colonization pattern of the gut, (ii) the functioning of the local immune apparatus and (iii) the response of the systemic immune system.

Three factors were distinguished:

(i) *antigen:* by measuring the colonization pattern of bacteria in the digestive tract (oropharynx and intestines) we gained an impression of the number of potentially pathogenic microorganisms in the alimentary canal together with the threshold value for translocation through the epithelium.

(ii) *local defence:* by determining the percentage of s-IgA coated aerobic Gram-negative bacteria in the faeces we received information concerning the function of the local immunity.

(iii) *systemic defence:* by determining circulating antibodies against potentially pathogenic (translocated) gut bacteria we received in combination with point (i) and (ii) information concerning the immunoregulating homeostasis, i.e., the T-cell function.

Sixty six patients with inflammatory bowel disease (36 with Crohn's disease and 30 with ulcerative colitis) were compared with a healthy control group. Three observations resulted from this investigation:

(i) qualitatively and quantitatively there was no difference in the potential pathogenic population of the digestive tract between IBD patients and healthy people.

(ii) in both the 'relapse' and 'remission' phases of the illnesses the patients showed a significantly lower function of the local immune mechanism compared with that of healthy controls.

(iii) the systemic immune mechanism showed an increased response to aerobic Gram-negative (translocated) gut antigens in comparison with the control group.

From the observations and from the evidence in the literature we concluded that a combined defence deficiency may exist in this type of

patient: a deficient local immune system in combination with a decreased T-suppressor cell activity.

If bacterial antigens play a role in the aetiology of these illnesses, then antigens from the anaerobic flora are probably important candidates, as many of this type of bacteria are present in high concentrations in the gut lumen (10^4 times higher than the aerobic *Enterobacteriaceae* species). Probably due to the combination of a deficient local immune mechanism and a decreased control over the immune response, the uptake of these antigens (which are formed close to the bowel wall) or even the translocation of certain anaerobic strains lead to increased concentrations of circulating antibodies. This condition could lead to allergy and/or granuloma formation.

At this moment, little perspective exists of treatment aiming at restoring or increasing the defence mechanisms. From our bacteriological point of view, it seems conceivable that both these chronic bowel diseases could probably best be treated by:

1. antigen elimination.
2. suppression of B-cell activity.

As it is unknown which anaerobic strains are important for the aetiology (should they play a role), elimination of those strains to which the patient possesses circulating antibodies should be considered. Practically this is difficult to achieve as the antigens must first be identified before undertaking this type of investigation. Therefore elimination of anaerobic antigens should mean: total decontamination of the digestive tract. The B-cells should be simultaneously depressed in the expectation of radically reducing B-cell clones specifically involved with the pathogenesis. When antibodies against the gut bacteria (*Eubacterium* species and *Peptostreptococcus* species) have been eliminated from the circulation then reconventionalization with anaerobic bacteria which support the colonization resistance may be carefully commenced. This must include no species to which the patient had circulating antibodies.

SAMENVATTING

Dit onderzoek behandelt enkele facetten der ontstaanswijze van de ziekte van Crohn en colitis ulcerosa, twee chronische ontstekingsziekten van het spijsverteringskanaal. De werkhypothese ging er van uit dat deze beide aandoeningen expressievormen van immuunreactiviteit ('overgevoeligheidreacties') zijn op antigenen normaal aanwezig in het lumen van het spijsverteringskanaal. Deze antigenen, mogelijk van microbiële (bacteriële of virale) of diëtische aard, zouden circulerende antistoffen doen kunnen ontstaan die kruisreageren met eigen weefsels; in dit geval van het spijsverteringskanaal. Ondermeer omdat voedsel waarschijnlijk weinig constant is van samenstelling en de koloniserende flora duidelijk meer, ging onze aandacht naar bacteriële antigenen van de endogene flora. Deze antigeenbron wordt, onder normale omstandigheden, onder controle gehouden door de afweer van de gast (locale en systematische afweer). De locale afweer poogt het antigeen binnen het lumen van de darm te houden door middel van het secretoir immuunglobuline A (s-IgA); het systematisch immuunapparaat (reagerend op endogene bacteriële antigenen met circulerende antistoffen) wordt onderdrukt door T-suppressor cel aktiviteit. Faalt deze immuunregulerende homeostasis dan zou verhoogde translocatie (d.i. passage vanuit het lumen naar de lym-

foide organen) kunnen optreden. Dit kan leiden tot circulerende antistoffen en mogelijk tot 'overgevoeligheidsreacties' (type 2 of 3).

Deze interactie tussen darmantigenen en afweer bestudeerden we in dit onderzoek met behulp van de aërobe *Enterobacteriaceae* spp, niet alleen als mogelijke allergenen maar ook als bruikbare indices voor het bepalen van het kolonisatiepatroon, van het functioneren van het locaal immuunapparaat en van de systematische immuunreactie. Drie faktoren werden onderscheiden:

(i) *antigeen:* door het kolonisatiepatroon van bacteriën in het spijsverteringskanaal (oropharynx en darm) te meten, kregen we een indruk van de hoeveelheid potentieel pathogenen in het spijsverteringskanaal en daarmee van de kans dat ze zouden kunnen transloceren door het epitheel.

(ii) *locale afweer:* door het percentage s-IgA gecoate aërobe Gram-negatieve bacteriën in de faeces te bepalen, verkregen we informatie over de functie van het locale immuunapparaat.

(iii) *systematische afweer:* door circulerende antistoffen te bepalen tegen potentieel pathogene (translocerende) darmbacteriën, verkregen we in samenhang met punten (i) en (ii) gegevens over de immuunregulerende homeostase, d.i. de T-cel funktie.

Zesenzestig patiënten (36 met de ziekte van Crohn en 30 met colitis ulcerosa) werden vergeleken met een gezonde controlegroep. Drie bevindingen kwamen uit dit onderzoek:

(i) kwalitatief en kwantitatief was er geen verschil in de potentieel pathogene populatie van het spijsverteringskanaal tussen IBD patiënten en gezonde personen.

(ii) zowel in de 'relapse' als in de 'remission' fase van de ziekte vertoonden de patiënten een significant verlaagde functie van het locaal immuunapparaat vergeleken met dat van de gezonde proefpersonen.

(iii) het systemisch immuunapparaat was verhoogd gesensibiliseerd tegenover aërobe Gram-negatieve (translocerende) darmantigenen, in vergelijking met de controlegroep.

Uit deze bevindingen en literatuurgegevens concludeerden we dat er een gecombineerde afweerstoornis zou kunnen bestaan in dit type

patiënt: een deficiënt locaal immuunapparaat in combinatie met een verminderde T-suppressor cel aktiviteit.

Indien een bacterieel antigeen een rol speelt in de ontstaanswijze van deze ziekten, dan zijn antigenen afkomstig uit de anaërobe flora waarschijnlijk belangrijke kandidaten. Immers, veel van deze bacteriesoorten zijn hoog geconcentreerd in het lumen van de darm aanwezig (10^4 hoger dan de *Enterobacteriaceae* spp). Waarschijnlijk dankzij de combinatie van een deficiënt locaal immuunapparaat en een verminderde controle over de immuunrespons kan opname van deze dichtbij de darmwand gevormde antigenen of zelfs translocatie van (bepaalde) anaërobe stammen leiden tot verhoogde concentraties van circulerende antistoffen. Dit zou tot 'overgevoeligheid' en/of granuloomvorming kunnen leiden.

Daar er op dit ogenblik weinig perspektief bestaat op afweer herstellende en/of verhogende behandelingen, menen wij bacteriologen dat, op grond van deze bevindingen, deze beide chronische darmziekten mogelijk het best behandeld kunnen worden door:

1. antigeen eliminatie.
2. suppressie van B-cel aktiviteit.

Daar niet bekend is welke anaërobe stammen — zo ze een rol spelen in de ontstaanswijze — van belang zijn , zou eliminatie kunnen overwogen worden van die anaërobe stammen waartegen patiënt circulerende antistoffen bezit en waarvoor hij overgevoelig is. Practisch is dit moeilijk uitvoerbaar daar voor zo'n onderzoek men het antigeen in handen moet hebben. Daarom zou eliminatie van anaëroob antigeen inhouden: een totale darmdeconcentratie. De B-cellen zouden gelijktijdig kunnen worden onderdrukt, in de hoop de specifiek bij de pathogenese betrokken B-cel kloon (klonen) sterk te decimeren. Zodra antilichamen tegen darmbacteriën (*Eubacterium* spp en *Peptostreptococcus* spp) uit de circulatie zijn verdwenen, zou dan voorzichtig met reconventionalisering met de voor de kolonisatie resistentie verantwoordelijke anaërobe flora kunnen worden begonnen. Hierin mogen dan geen species voorkomen waartegen patient circulerende antilichamen had.

With special thanks
To my grandfather Mr. F. van Saene
To my aunt Mrs. J. Beeckman
To my parents Mr. & Mrs. van Saene-Beeckman
To my sister Magda and her husband Paul
To my brother Joris
To my two nieces Sarah and Karen
And to you, lovely girl friends. That I am happy to-day to present this thesis is due to your support, encouragement, indulgence and patience
But what should be my happiness, if I could not share it with you all my very good friends. Your affection your true friendship I value above all
I thank you very, very much indeed
Especially, to Alfred Sachs and Pieter de Graeff
I am proud of you, yo' the bess
Personal thanks to the inflammatory bowel disease team Marisca de Zwaan Gé Helmus, Margriet Wiersma, Jaap Krikken and Edith van Santen
I never forget you Jaap and Edith, you performed most of the experiments on which the original observations are based
Your technical work was superb and if this investigation will be of value in the future to you all the merits
However, the analyses would not have been possible if the nursing staff of the department of gastroenterology had not been so neurotically cooperative in obtaining specimens of the 66 patients during the period of three and half years, especially personal thanks to Ria Tepper, you take a warm place in my heart
To Willy Jansen for her helpful criticism mainly on clinical matters during the preparation of this manuscript
To Vasek Fidler for his help regarding the statistical analysis even at the most inopportune times
To Els Stuut and Bert Schaalma for their excellent bibliographic service
To Wil Noordik, who helped me with the original artwork
To Jack Duitsch for his impeccable photography
To Alex Lande and Isabel Brown for correcting the language with intelligence and precision
To the secretaries Mrs. M.H. Brummer-Scholten, Annigje Dijkstra and Sjoerdtje Swart for deciphering my hieroglyphics and typing this manuscript
To Geert Verstaen and Geert Seynhaeve of Lannoo, who was most helpful at all stages of the preparation of this monograph
To van der Waaij for giving me my start, who constantly encouraged me who never left me during this study, who has sacrified so much for me

I thank you, Dick.